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Efeitos de várias propriedades do solo em testes ecotoxicológicos

Effects of various soil properties on ecotoxicological testing

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To my dear Hugo, for you have been my day light, my sweet cherry!

To LIFE!

resumo

Estudos na área da ecotoxicologia de solo são normalmente realizados em solos padrão, tais como o solo artificial OCDE ou o solo natural LUFA. Ao avaliar os efeitos de tóxicos no ambiente, as propriedades dos solos são frequentemente diferentes das dos solos padrão, o que pode conduzir a uma situação de exposição diferente para as espécies teste e, portanto, a conclusões erradas. Com base no conceito dos “Euro Solos”, seis diferentes tipos de solos, seleccionados de forma a incluir uma grande variedade de propriedades, foram estudados em relação à sua adequabilidade para quatro espécies teste: *Enchytraeus albidus*, *Enchytraeus luxuriosus* (Enchytraeidae), *Folsomia candida* e *Hypogastrura assimilis* (Collembola). Nos testes de reprodução, observou-se que as espécies teste reagiram de forma muito diferente aos solos (p.e., os dois enquitraídeos não sobrevivem em solos ácidos). *F. candida* é menos afectada pelas propriedades do solos. As diferenças encontradas estão relacionadas com o tipo de solo utilizado, cujas propriedades diferem consideravelmente. Nos testes principais, o efeito da substância teste referencia Phenmedipham (formulação Betanal) e o metal pesado cobre (na forma de $CuCl_2 2(H_2O)$) foram estudados nos solos em que ocorreu reprodução, avaliado previamente. Os resultados mostram que as espécies teste reagem de forma diferente nos vários solos e à substância aplicada, indicando uma interacção entre as diferentes propriedades do solo e as substâncias teste. O tema da diversidade de solos e consequências para os organismos e procedimentos teste são problemas que ainda não possuem solução na totalidade. No entanto, foram dados passos importantes no sentido de obter uma solução e/ou alertar para este problema.

abstract

Soil ecotoxicology studies are usually performed in standard soils, such as OECD artificial soil or LUFA, a natural soil. When assessing the toxic effects in the environment, soil properties are often different from those in standard soils, which might lead to a different exposure situation for the test species and, therefore, to misleading conclusions. Selected to cover a broad range of properties and based on the Euro Soils concept, six different soil types were studied regarding their suitability to four test species: *Enchytraeus albidus*, *Enchytraeus luxuriosus* (Enchytraeidae), *Folsomia candida* and *Hypogastrura assimilis* (Collembola). In the reproduction tests, it was found that the test species reacted very differently to the soils (e.g., the two enchytraeids do not survive in acid soils). *F. candida* is less affected by soil properties. The differences found are related with the soil properties which differ considerably between the studied soils. In the main tests, the effect of the reference test substance Phenmedipham (formulation Betanal) and the heavy metal copper (as $CuCl_2 2(H_2O)$) was studied in those soils where sufficient reproduction was determined beforehand. Results show that the test species react differently to the various soils and to the applied substance, indicating an interaction between the different soil properties and the test substances. The issue of soils diversity and consequences for organisms and testing procedures is a problem yet to be solved in its completeness. Nevertheless, steps were made in the direction of finding a solution and/or alert to this problem.

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Chapter 1

General Introduction

1. General Introduction

1. Ecotoxicology – an Introduction

The science of ecotoxicology is an outgrowth of the link between toxicology, ecology and chemistry. The term “ecotoxicology” was coined by Truhaut in 1969 (Butler, 1984), when the scope of investigation on the harmful effects of substances switched from a single organism (humans) to entire ecosystems. Ecotoxicology became necessary when human beings began introducing chemicals into the environment. No one was concerned about the fate of these chemicals until they turned up in unexpected places or had adverse effects on organisms in the environment (Römbke and Moltmann, 1996). The task of ecotoxicology is to assess, monitor and predict the fate and effects of foreign substances in the environment (Moriarty, 1988).

This paper mainly focus on the environmental compartment soil. Many soil systems worldwide have been contaminated with chemicals and require some form of mitigation or remediation. However, soil systems are essential for sustaining agricultural practices, perpetuating natural resource industries such as timber production, and maintaining the proper functioning of natural ecosystems. To maintain soil quality and protect human health and the environment from past and current additions of chemicals, it is imperative that we understand the effects of chemicals on soil organisms and develop a sound regulatory strategy for the protection of soils (Lanno *et al.*, 2003).

Laboratory ecotoxicity tests were developed to evaluate and predict effects in the environment. These effects can be of short term duration, such as mortality (assessed by the acute tests), or long term (in small organisms most of the individual life-cycle, in larger animals, up to one third of the mature life cycle) in the case of sublethal parameters (e.g. reproduction, growth), assessed by the chronic tests. Moreover, these two kind of tests also differ in terms of their orientation: acute tests are usually

employed as a "screening tool" with high concentrations over a broad range, whereas chronic procedures tend to be applied for in-depth investigations, i.e. concentrations are used which do not have any acute effects, or only a low mortality, in the preceding acute toxicity test (Hamburger, 1983). Some substances have an immediate, lethal effect, whereas others may have impacts which do not become manifest until later, e.g. when a population declines as a result of reduced fertility (Römbke and Moltmann, 1996). The kind of effect that is measured is of different importance and risk assessment criteria must be adjusted accordingly.

There are several organisms that are commonly named as "test organisms" due to the fact of being frequently used to assess the effect of toxic substances. These organisms have been chosen due to several reasons but the following pre-requisites are essential: the test species have to be easy to handle, have a short life-cycle, and reproduce well in laboratory. At the soil level, the earthworm acute test (OECD, 1984) is the oldest and still the most common test preformed. Species diversity of soil organisms is very high but, by necessity; risk assessment of the effects of chemicals on soil biota has to be restricted to only a few of these organisms. Thus, appropriate selection of the test species is an indispensable prerequisite for a good risk assessment system. Test organisms should be representative in terms of function, taxonomy, trophic level, life-history strategy and route of exposure to toxicants (Laskowski *et al.*, 1998).

On the contrary to the aquatic compartment, soil ecotoxicology is a young science, and as a consequence, there was a lack of standardised laboratory toxicity tests with soil organisms for a long time (Van Gestel, 1998), but the situation is improving. At present, acute and chronic tests with earthworms, collembolans and enchytraeids were standardised by OECD (for single chemicals) and ISO (for soil quality assessment). Two test species can be seen in Figure 1. Some other organisms are on the way to become a standard test species (e.g. the predatory mite *Hypoaspis aculeifer*; Bakker *et al.* 2003)).

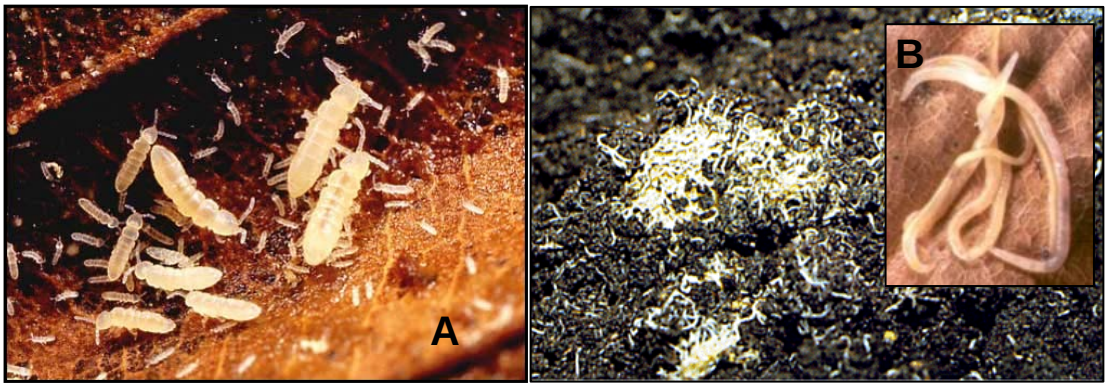

Figure 1: **A**- Group of individuals, adults and juveniles, of the species *Folsomia candida* (the largest adult is 2 mm in length). **B**- Culture of *Enchytraeus albidus* plus some individuals (length about 1 cm) enlarged (inlet).

Standard Toxicity Tests follow strict protocols so that they can be performed elsewhere in the exact same way. In this way, several items have to be fixed in detail: laboratory cultures of the test organisms, test conditions, test medium, all have to follow exactly the standard. For instance, the OECD artificial soil (OECD guideline N. 207, 1984), is constituted of 70% quartz sand, 20% kaolin clay and 10% peat, with a pH value adjusted to 6 ±0.5 by addition of $CaCO_3$. Although these specifications for an artificial soil are very important in to compare test results between laboratories, the representativity and variety of natural soils is completely underestimated.

2. Background on Soil

Soils are a part of terrestrial ecosystems. The bedrock, the regional climatic conditions and the influence of biological factors determine whether and what types of soils evolve (Römbke and Moltmann, 1996). Consequently, there is extremely wide variety of soil types with significant differences in composition and characteristics, which are constantly changing, even without human influence (ECETOC, 1990). Therefore, there is a huge amount of different soil types, which have different properties causing different interactions with the chemicals and

organisms. According to Zagury *et al.* (2002), the main soil variables involved in the mobility of contaminants are: pH, oxidoreduction potential (ORP), organic matter (OM), clay mineral, hydrous oxide, carbonate and salt contents. For example, carbonates, exchangeable cations and OM will contribute to the soil buffer capacity whereas clay minerals, OM and salt content will influence the cation exchange capacity (CEC). Buffer capacity gives an indication of the soil resistance to a pH variation. CEC (usually expressed as milliequivalents per 100 g of soil) is defined as the sum of the exchangeable cations of a soil. Changes in soil environment conditions may enhance or diminish contaminant mobility by changing its chemical form. Additionally, soil biological activity may be responsible for important changes in environmental conditions.

Once the nature of the soil and the physicochemical properties of the contaminant are known, it is possible to assess the distribution of the contaminant between the different phases of the soil. The non-polarity of some **organic contaminants**, like the petroleum hydrocarbon molecule, permits only a weak interaction with the clay particle surfaces (van der Waals). However, in the case of positively charged organic molecules (by protonation), it is expected that they will be adsorbed on the soil (clay) solid surfaces, depending on the CEC of the soil. The pH of the medium will interfere with ionisable organic compounds, like phenols, by greatly influencing their aqueous solubility. Nevertheless, the main recipient of the organic contamination is the organic matter. It has been shown that organic matter is composed of dense and enlarged compartments (Young and Weber, 1995). The enlarged OM absorbs organic contaminants, but desorption from its surface is fast and easy. The dense fraction absorbs contaminants much more strongly and the desorption is diffusion-limited. The process of aging (i.e. a time period of intensive interaction between soil particles and contaminant) results in an increase in the fraction of contaminants sorbed to this dense compartment (Luthy *et al.*, 1997). Nevertheless, the fraction of total organic matter in the soil helps to evaluate the soil-water distribution coefficient of hydrophobic organic contaminants.

In the case of **heavy metals** in soils, these can be found in several pools:

a) Dissolved in the soil solution (as either free ion or a soluble complex);

b) Occupying exchange sites on inorganic soil constituents;

c) Specifically adsorbed (short-range chemical forces such as ionic or covalent bonding) on inorganic soil constituents;

d) Associated (complexation or adsorption) with insoluble soil organic matter or organic colloids;

e) Present in the structure of primary and/or secondary minerals.

Soil particulate phases frequently have high concentrations of metals relative to concentrations of metals in the dissolved phase. The concentration in the soil solution is governed by a number of interrelated processes, including inorganic and organic complexation, oxidation-reduction, precipitation/dissolution reactions and adsorption/desorption reactions (McLean and Bledsoe, 1992). Metal speciation or partitioning rather than total concentration is the key to understanding its mobility and its potential bioavailability and toxicity.

In sum, the manner in which contaminants interact with soil properties and organisms is central to understanding their fate, transport, bioavailability and, finally, the occurrence of effects.

3. Aims

The main objective of this thesis is to understand the way soil properties interact with soil organisms and chemical substances. In this way, it was intended to adapt the existing ecotoxicological standard procedures for European field soils. The results of this study will help to improve the risk assessment of chemical substances in soil or contaminated sites.

To fulfil the main aim of this thesis, three steps were performed:

I. Soil selection and characterisation of representative European field soils, based on the "EURO-Soils" concept (Kuhnt & Muntau,1992), addressed in Chapter 2.

II. Investigation of the selected soils (reproduction level) with two groups of organisms, enchytraeids and collembolans, to evaluate their sensitivity towards the different soil parameters presented in Chapters 4, 5 and 6.

III. Testing of the selected soils spiked with two different chemical substances (laboratory mono-specific contamination with Phenmedipham and Copper chloride) with the two groups of organisms, in Chapters 5, 6 and 7.

Additionally, a review on the ecological requirements of the main standard test species in soil was performed to evaluate what is known to influence the test species (in Chapter 3). Finally, a novel approach to assess the effects of chemicals in soils is proposed, i.e. using the avoidance behaviour of enchytraeid as a screening test (in Chapter 4).

References are presented at the end of each chapter and according to the publication requirements of the journal where the papers were published/submitted.

4. Details on materials and tests performance

Based on the EURO-Soils (ES) approach, soils were selected to perform the toxicity testing in a natural medium, rather than just in the standard soils such as the artificial OECD or natural LUFA 2.2 soil. The respective natural soils having the same main properties as the original EURO-Soils are termed SIM-Soils. The following figure is only to give a visual idea of how different soils can look.

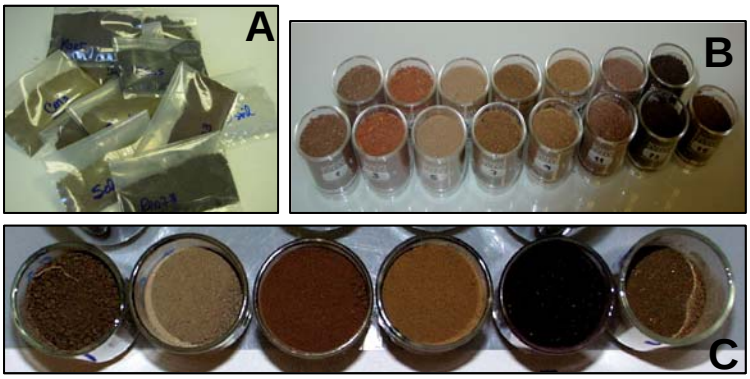

Figure 2: Test soils: **A**: Soils divided in plastic bags for storing. **B**: SimSoils in vessels. **C**- Euro-Soils in vessels (from left to right: ES1, ES2, ES3, ES4, ES5, ES7).

The test procedures of the reproduction tests followed several main steps (figures 3 to 8), as follows:

1- Contamination of the test soils with the test item;

2- Contaminated soil is divided into the test vessels;

3- Introduction of the organisms into the test vessels – test start;

4- Test is running for a certain amount of time (e.g., 6 weeks for *Enchytraeus albidus*);

5- Test ends.

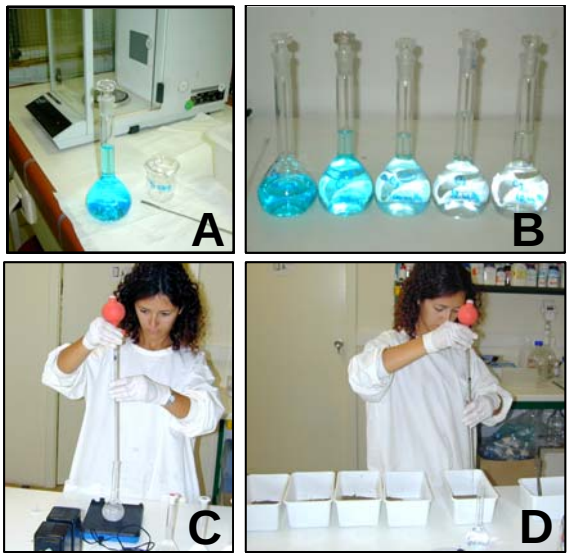

Figure 3: Preparation of the chemical solutions. **A-** Weighing the chemical for the stock solution. **B-** Dilutions of the chemical solutions. **C** and **D-** Addition of the chemical solution to each pre-moistened soil batch.

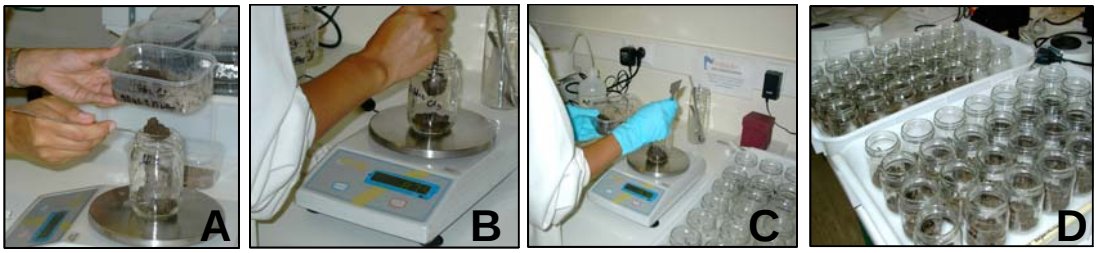

Figure 4: Test vessel preparation. **A**, **B**, **C**- Introduction of the test soil (control or contaminated) into the test vessels in the correct amount. **D**- Group of test vessels prepared for the test start.

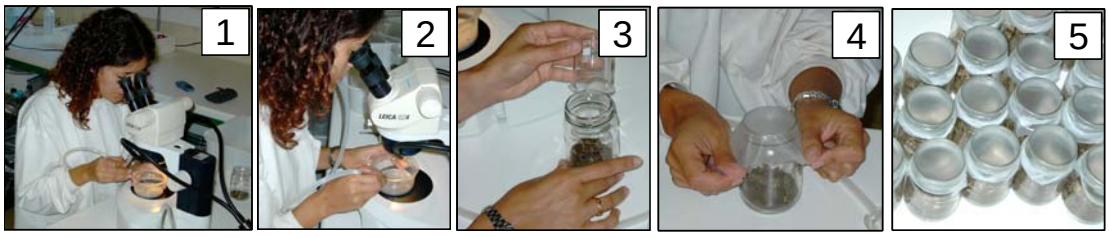

Figure 5: Collembolan test start. **1** and **2**- Selection of the collembolans from the culture boxes and collection into a small plastic vessel. **3**- Introduction of the organisms into the test vessel. **4**- Placement of the parafilm lid on the test vessel. **5**- group of prepared test vessels

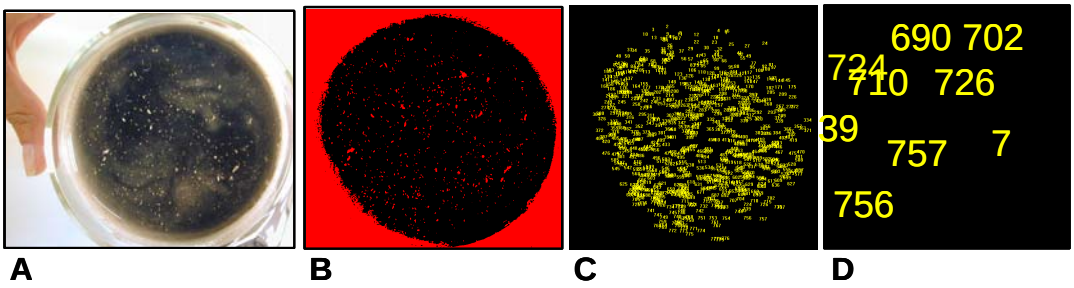

Figure 6: Collembolan test end. **A-** Collembolans floating on the water surface (plus black ink) after the test vessel being filled with water and stirred for the organisms release from the soil particles. **B-** Transformed picture, from Sigma Scan Pro 5 software program, in which the white spots (*F. candida*) are coloured as red **C-** Automatic counting of the previous red spots. **D-** Detail of C.

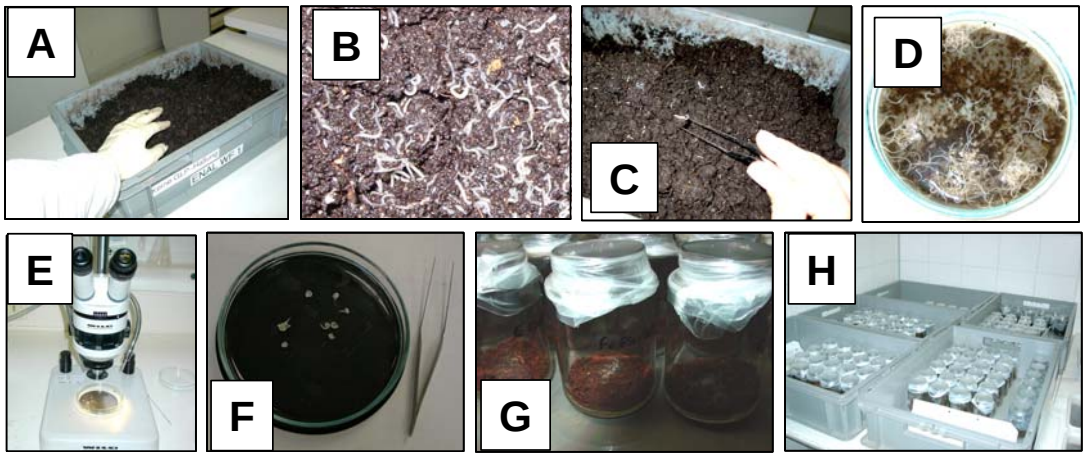

Figure 7: Enchytraeid test start. **A-** Laboratory culture of *E. albidus*. **B-** Detail of A. **C-** Collection of individuals for the test. **D-** Petri-dish filled with deionised water and enchytraeids. **E-** Visualisation of the organisms under the binocular for selection of adults with eggs in the clitellum. **F-** Organisms selected for the test start. **G-** Test vessels with the organisms plus food supply and lid. **H-** Several tests running under test conditions.

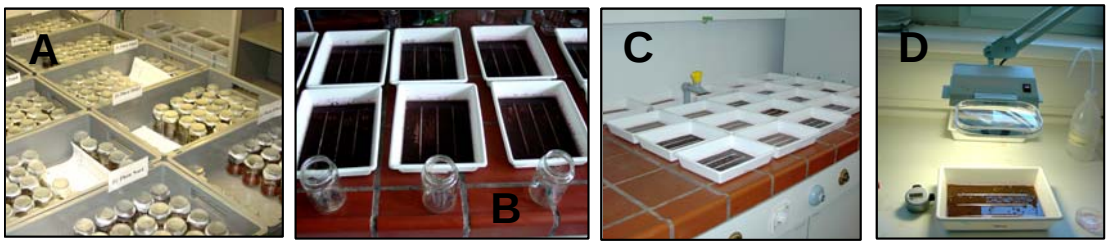

Figure 8: Enchytraeid test end. **A**- Group of tests running (final day). **B**- Test vessels were filled with alcohol and Bengal red. Afterwards the test substrate of each vessel was transferred into flattened boxes to spread the soil and organisms. **C**- Boxes under a fume hood for some hours in order to allow the colouration of the organisms and the soil to settle down. **D**- Observation of the boxes under a binocular for counting procedures.

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Chapter 2

Tackling the heterogeneity of soils in ecotoxicological testing: An EURO-Soil based approach

2. Tackling the heterogeneity of soils in ecotoxicological testing: An EURO-Soil based approach

Jörg Römbke & Mónica Amorim

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ABSTRACT

Goal, Scope and Background: In terrestrial ecotoxicology, standardised test methods using plants, earthworms and insects are available for the evaluation of effects induced by heavy metals, organic chemicals and, in particular, pesticides. Currently, these tests are performed either by using so-called Artificial Soils or (more or less) arbitrarily selected natural soils. Consequently, the test results depend not only on the intrinsic physico-chemical properties of the test chemical but are also significantly influenced by the variable properties of the chosen soil. In order to standardise the test conditions and, at the same time, to relate the test results to representative soil types within the EU, it is proposed to modify the EURO-Soil concept for testing purposes.

Main Features: The EURO-Soil concept, i.e. the selection of a limited number of soils that are representative for Europe, was originally developed for the performance of standardised environmental fate tests. Despite many problems in detail, soils from six sites all over Europe were identified that cover a wide range of soil properties (e.g. texture, pH, organic matter content) and, therefore, very different conditions concerning the bioavailability and, in turn, the effects of chemicals. Obviously, the routine use of EURO-Soils as a control or test substrate would require large amounts of soil. Therefore, it is proposed to modify this concept in a way such that for ecotoxicological tests all soils similar to one of the six EURO-Soils can be used.

Results and Discussion: It is assumed that that the six EURO-Soils are representative for wide areas of the European Union but at the same time it is neglected that some soils typical for, e.g. Northern Scandinavia, have to be identified in the future. All soils having similar properties (i.e. texture, pH, C/N ratio, and organic matter content) as one of the original EURO-Soils are called SIM-Soils. In this contribution "ranges" are proposed for four main properties and the six EURO-Soils, thus allowing the identification of the SIM-Soils. However, since these properties cover a continuum soils cannot be classified easily into a small number of classes; expert knowledge is required in order to decide whether a natural soil belongs to a certain SIM-Soil class or not. In the long run, this classification must take biological parameters like their suitability for standard test organisms into account.

Recommendation and Outlook: The soils selected so far (at least one for each EURO-Soil) are actually tested using different biological test methods. Further tests are necessary in order to decide which tests can be done in which soil and whether new test systems, e.g. covering acid soils, have to be developed. However, it is already clear that the standard test species differ distinctly regarding their sensitivity towards soil properties. It is recommended to use the SIM-Soils in order to provide the authorities with more field-relevant data when assessing chemicals in the terrestrial environment.

Keywords: Texture, pH, organic matter content, C/N-ratio, natural soils, environmental risk assessment

1. Introduction

In terrestrial ecotoxicology, standardised test methods using plants, earthworms and insects are available for the evaluation of effects induced by heavy metals, organic chemicals and, in particular, pesticides (Römbke & Knacker 2003). The first of these methods, describing the study of acute effects on earthworms (OECD 1984), introduced the idea of using an artificial soil as test substrate. It consisted of a mixture of sand (70%), kaolinite clay (20%), grounded peat (10%), calcium carbonate (in order to get a pH of 6.0 ± 0.5) and water (Tab. 1; please note that the individual values can change slightly according to the batch used). This composition mainly reflects two ideas: firstly, it must be suitable for the compost worm *Eisenia fetida*, the first standard test species in soil, which clearly prefers substrates with high organic matter content. Actually, it is not considered to be a "true" soil inhabitant, but on the other hand is one of the very few earthworm species to be kept in mass cultures (Edwards & Coulson 1992). Secondly, the test substrate should be easily mixed in a standardised way. For this reason the components were selected for general availability, preferably globally. In fact, with the exception of the organic matter this is true – and it seems that even the latter can be substituted with local sources (for an example from the tropics see Garcia (2004)).

While this substrate is not considered to be a real soil by soil scientists (e.g. because it lacks the typical porous structure of natural soils), it has become extremely popular among ecotoxicologists. In fact, most of the terrestrial tests using invertebrates today are performed with this artificial substrate (often referred to as "OECD soil"). For example, besides existing tests with earthworms (*Eisenia fetida*, *E. andrei*), enchytraeids (*Enchytraeus albidus*, *E. crypticus*) and collembolans (*Folsomia candida*) still new tests using artificial soil are under development (e.g. with the herbivorous beetle (*Oxythyrea funesta*, ISO 2002)). Even plant tests can easily be performed with OECD soil (e.g. Kalsch & Römbke 1999), but usually they are performed with field soils. However, these soils are defined very superficial, since only their amount of organic matter (< 1.5%) is specified (OECD 2003). Depending on the respective plant test species a suitable soil is sometimes difficult to find.

The basic idea of using an artificial substrate based on a small number of (more or less) well-standardised components was implemented for sediment tests (Egeler *et al.* 1997). However, at the same time artificial soil has also heavily been criticised from an exposure-orientated point of view. It is argued that due to the high amount of organic material the bioavailability of many organics (plus some heavy metals) is lowered in comparison to natural soils, meaning that the risk of a chemical is underestimated when basing the risk assessment on data from tests with artificial soil. However, while it is true that in many cases the toxicity of a certain chemical is lower in artificial soil than in natural soils; this is not always the case (e.g. for Collembolan tests). However, the growing critics support the idea that a natural soil should be used as the standard test substrate (e.g. Van Gestel & Weeks 2004).

Table 1: Physical-chemical properties of OECD artificial soil and LUFA St. 2.2 standard soil (Garcia, 2004).

Parameter	**Unit**	**OECD Artificial Soil**	**LUFA 2.2**
Texture (sand, silt, clay)	%	80 - 10 - 10	77 - 17 - 6
pH ($CaCl_2$)		6.1	6.1
P	mg/dm^3	1	324
K		10	49
Na		64	22
Ca	c.molc /dm^3	6.29	10.54
Mg		0.55	0.05
N total	%	0.11	0.19
C org		3.59	2.70
Organic material		6.17	4.64
C/N ratio		32.6	14.2
Fe	mg/dm^3	9	288
Zn		1.30	31.9
Mn		0.41	84
Cu		0.02	8.52
Density	g/cm^3	1.1	1.2
WHCmax	%	56.1	50.0

Obviously the number of natural soils potentially suitable for ecotoxicological testing is so high that the results of such tests would be impossible to compare and thus the data would be useless for regulatory purposes. So, the first step in this process must be a reduction of possibilities. In this situation, soil ecotoxicologists have taken over an idea first brought up by chemists studying the fate of pesticides in soil: the testing of just five soils, more or less representative for agricultural soils, but differing in organic matter content and texture. The OECD (1981) proposed this idea as part of its adsorption/desorption test guideline. A similar but slightly modified approach recommending a lower (three) number of soils was later proposed for Germany too (Schinkel 1985). These soils were provided by the Agricultural Research Station in Speyer (in German: Landwirtschaftliche Forschungsanstalt, abbreviated LUFA) and thus are known as LUFA soils. Later on, during a workshop in Belgirate (Italy) OECD combined these ideas in order to bring them in line with the EURO-Soil concept (see chapter 2; OECD 1995). Finally, the suitability of the most often-used of these LUFA soils (No. 2.2; Table 1) and the relationship of results gained with this soil compared to data from artificial soil tests was studied in the sizable EU project SECOFASE with three chemicals (dimethoate, LAS, copper) and about a dozen test species (Løkke & Gestel 1998). However, while these soils are widely accepted for the investigation of the fate of chemicals (e.g. degradation, metabolisation, leaching), LUFA St. 2.2 was not recommended in international test guidelines, maybe because the selection of this soil was artificial: on an international scale it is incomprehensible that exclusively this soil should be the standard test substrate.

In this situation, the idea to use the EURO-Soil concept also for ecotoxicological effect testing came up. So, at the same time, the test results could be related to representative soil types within the EU. However, due to problems related to this concept it cannot be realized without modifications. In this contribution such a modified concept is set up for discussion. Comments are explicitly appreciated.

2. The EURO-Soil concept

The EURO-Soil concept was developed in the context of the implementation of the OECD Test Guideline 106 (Adsorption/Desorption), since the test results obviously depend not only on the intrinsic physico-chemical properties of the test chemical but are also significantly influenced by the variable properties of the chosen soil. So, the main aim was the selection of **Representative Soils** which reflect the major properties of wide areas of Europe. In addition, it was intended to establish the **Standard Operating Procedure** for the selection of sampling sites, treatment and characterisation of soil samples, and **Distribution Models** that are able to simulate and predict the adsorption/desorption of chemicals (in particular pesticides) in quantitative terms (Kuhnt *et al*, 1999). Due to this background of fate research the EURO-Soils were selected in such a way that a wide range of sorption-controlling soil properties were covered.

After reviewing the available information on soils of the European Union (EU) in its size at that time (i.e. without the Iberian peninsula, Scandinavia and Eastern Europe), six sites were selected in the following regions: Sicily (Italy), Peloponnesus (Greece), Wales (UK), Normandy (France), Schleswig-Holstein (Germany) and, some years later than the first five ones, Lungau (Austria). The soils selected were chosen in such a manner that the entire set of properties reflects in a representative way the most frequent and typical soil types of the EU (Kuhnt and Muntau, 1992; Gawlik *et al.* 1996). At each site soil samples were taken and analysed twice. After being treated (e.g. sieved to 2 mm, dried and defaunated) the soil samples were stored in large amounts. Their main properties are described in Table 2. Please keep in mind that due to the fact that samples were taken twice and measured even more often, values for the different properties vary slightly in the literature.

Despite the fact that the EURO-Soils had been especially designed for the purpose of creating a representative soil set for adsorption/desorption testing and other fate tests (OECD 1995, Gawlik 1998, Gawlik *et al.* 2003), they were considered for a broader application soon after their introduction in 1990 (Gawlik *et al.*, 2001). For example,

the original sampling sites have been used as reference sites for ring tests measuring soil properties like the pH. In addition, the soil samples serve as reference material for validation of residue analytical methods (only small amounts of soil are necessary for this purpose). Such reference materials are gaining more and more importance in the context of the Commission's environmental policy (Gawlik *et al.* 2003). However, with one exception (their use as substrate in microbiologial tests has been discussed at the Belgirate workshop (OECD 1995)) there was only one attempt to use the EURO-Soils for routine ecotoxicological effect testing: at a SETAC workshop it was proposed to use two specifically for assessing the hazard of cationic and anionic heavy metals (Fairbrother *et al.* 2002). As an example the soils appropriate for invertebrate tests ES3 and ES2 or ES4 are mentioned (Løkke *et al.* 2002).

Table 2: Summary table describing some properties of EURO-SoilS. For details see Weissteiner *et al.* (1999).

Pedologic Parameters	**ES 1 Italy**	**ES 2 Greece**	**ES 3 UK**	**ES 4 France**	**ES 5 Germany**	**ES 6** [a] **France**	**ES 7 Austria**
Sand total	3	3	46	4	81	2	46
Silt total	22	64	37	76	12	82.4	35
Clay total	75	23	17	20	6	16	19
CEC	299	283	183	175	327	114	49.8
pH (H_2O)	6.0	8.0	6.0	7.0	4.6	8.3	5.2
pH ($CaCl_2$)	5.0	7.4	5.0	6.5	3.2	7.2	4.4
C tot	1.5	10.9	3.7	1.7	10.9	0.3	6.9
C org	1.3	3.7	3.45	1.55	9.25	0.25	6.7
Organic Matter	2.65	6.40	6.45	2.85	15.90	0.8	11.7
C/N ratio	7.6	18.5	13.3	9.7	30.7	12.5	14.3
FAO soil unit	**Vertic Cambisoil**	**Ortic Rendzina**	**Districc Cambisoil**	**Ortic Luvisoil**	**Ortic Podzol**	**BC Horizon of ES4**	**Districc Cambisoil**
Land use form	Grassland	Forest	Grassland	Crop site	Forest	-	Grassland

[a] Since **ES6** is the BC horizon of ES4, it will not be discussed anymore.

This hesitation to broaden their use in biological tests is caused by several facts:

- As mentioned earlier, most test species have been used in tests with artificial soil or a few natural soils. Therefore, it is simply not known whether they can be used in a wide range of natural soils with their considerably differing properties.
- In ecotoxicological tests a lot of substrate is needed (for example, in an earthworm reproduction test 12 kg dry weight). Any specific sampling site would

quickly be “empty” if it should provide the substrate for the many invertebrate tests done currently in Europe.

The first problem can be resolved by studying the behaviour, in particular the reproduction, of the standard test species in the EURO-Soils (or an even broader range of natural soils). In fact, such research is currently going on (in Europe: e.g. Amorim *et al.* (2004a) and in North America (Stephenson and Feisthauer 2003)).

The second problem cannot be solved by using the EURO-Soils for ecotoxicological effect testing as currently defined. In addition, some of the original EURO-Soil sites have been considerably changed in the meantime. Therefore, a modified concept is proposed which attempts to refer to the same basic considerations but also tries to overcome the practical problems in the following chapter.

3. Selection and characterisation of soils similar to a EURO-Soil

Since the EURO-Soils are not an endless resource, it is proposed to modify the initial concept as follows:

1. The selection of the original EURO-Soils is accepted, i.e. the fact that six soils are representative for the area of the (old) European Union. Very probably some more EURO-Soils have to be identified in order to cover those regions which recently joined the EU (e.g. an acid soil from a North Scandinavian site).

2. Instead of using soils from the original sites for routine testing, it is proposed to classify natural soils from all over the European Union according to the main properties of the original six EURO-Soils. As main properties the following have to be used: texture, pH, organic matter, and C/N-ratio. These properties are ecologically relevant because they determine to a large extent the occurrence of soil organisms. In addition, the same properties largely determine the fate of chemicals in soil.

3. All soils having the same properties as one of the original EURO-Soils may be called "SIM-Soils". In order to facilitate working with them each of these soils could be designated with a codename which refers to its real location as well as the EURO-Soil it is similar to (e.g. by adding an ESx).

4. However, before doing so, the question "What is similar?" has to be answered. It is rarely the case that a natural soil has exactly the same properties as a EURO-Soil. Therefore, a decision has to be made as to how large a certain range for each main property should be. Since there is no accepted method to define such ranges, "expert knowledge" was used to determine upper and lower values. In addition, for certain properties the classification as done by the German Federal Institute for Geological Sciences was applied (AG Boden 1996). The result of this exercise is shown in Table 3. Please note that the SIM-Soil classes do not intend to cover all possible combinations or all real natural soils.

However, initially this is merely a proposal. So the endeavour was to find natural soils belonging to each EURO-Soil class in reality.

Table 3: Selected acceptance ranges proposed for soils to be similar to an existing EURO-Soil (Gawlik 1998, Weissteiner *et al.* 1999).

EURO-Soil	**ES1**	**ES2**	**ES3**	**ES4**	**ES5**	**ES7**
pH ($CaCl_2$)						
Measured	5.1 - 5.7	7.2 - 7.5	5.2 - 5.9	6.5 - 6.8	3.2 - 3.2	3.5-4.5
Accepted range	5.0 – 6.5	6.5 - 7.5	5.0 - 6.5	6.5 - 7.5	3.0 - 4.0	3.5 - 4.5
Organic Matter (%)						
Measured	2.2 - 2.7	4.1 - 6.4	5.7 – 6.5	2.3 - 2.7	7.6 - 15.9	11.5
Accepted range	2.0 - 4.0	4.0 - 8.0	4.0 - 8.0	2.0 - 4.0	> 8.0	>8.0
C/N ratio						
Measured	8.8 - 11.3	47.4 - 54.5	11.6 - 14.2	10.6 - 11.3	31.9 - 36.3	14.2
Accepted range	< 10	> 20	10 – 20	10 - 20	> 20	10 – 20
Texture (%)						
(Sand-Silt-Clay)						
Measured	3 – 22 - 75	13 - 64 - 23	46 – 37 - 17	4 - 76 - 20	81 - 13 - 6	46 - 35 - 19
Accepted range	Clay	Silt	Loam	Silt	Sand	Loam

4. Identification of SIM-Soils

After the ranges of accepted values had been set, the search for soils took place. Due to logistic reasons mainly soils from Germany and Portugal were selected. Among the dozens of soils found in soil maps or similar sources, eleven soils fit with one of the original EURO-Soils (Tab. 3), while two soils could not be classified. In this case "fitting" means that three out of four measured properties must fall into the range defined for each SIM-Soils class. Large amounts of these eleven soils were prepared for storing, characterisation and testing as follows: dried at room temperature, sieved to 2 mm and defaunated by freezing (-30ºC) and unfreezing three times.

Table 4: Main properties of the selected SIM-Soils in comparison to the properties of the original EURO-Soils. In addition to the main properties, CEC and WHC_{max} are added in order to characterize the SIM-Soils.

SOIL	pH ($CaCl_2$)	O.M. (%)	C/N	Texture (%)	CEC [a] mval/100g	WHC (%)
ES1 Recommended	***5.0 – 6.0***	***2.0 – 4.0***	***< 10***	***Clay***		
Nat1	6.2	1.7	8.7	Clay	40.7	58.4
ES2 Recommended	***6.5 – 7.5***	***4.0 - 8.0***	***> 20***	***Silt***		
Hoh2	6.2	12.9	25.0	Silt	78.3	73.9
ES3 Recommended	***5.0 – 6.0***	***4.0 - 8.0***	***10.0 – 20.0***	***Loam***		
ESo3	5.2	6.0	11.8	Loam	74.5	
Sch3	5.4	4.1	10.4	Loam	68.5	67.4
Coi3	6.7	6.5	17.0	Loam	75.8	68.1
LUFA 2.2	5.8	4.4	14.0	Sandy loam	11.2	55.0
ES4 Recommended	***6.5 - 7.5***	***2.0 - 4.0***	***10.0 – 20.0***	***Silt***		
Mon4	6.5	2.5	9.7	Silt	20.7	53.2
Tau4	6.9	2.9	9.7	Silt	61.3	63.1
ES5 Recommended	***3.0 - 4.0***	***> 8.0***	***> 20***	***Sand***		
ESo5	3.2	9.2	29.7	Sand	87	100.1
ES7 Recommended	***3.5 - 4.5***	***>8.0***	***10.0 - 20.0***	***Loam***		
Ren7	3.8	8.7	11.0	Loam	132	121.8
Soils not classified into ES1 – ES7						
EsoX	6.3	8.9	23.5	Loam	?	64.0
KarX	3.7	10.6	45.9	Silt	173	72.0
OECD Soil	6.0	8.0	Ca. 40	Artificial soil	45.8	Ca. 90

[a] In some cases the CEC values were difficult to determine; therefore the values should be used with care.

The name codes are defined as follows: ES means EURO-Soil, ESo means that the soil is a sample from the same site, where the original EURO-Soil came from. In the other cases, three letters refer to the first three letters of the name of the sampling site while the last number signifies that the soil is similar to a certain ES number: Nat1: Natzungen; Hoh2: Hohenlimburg; Sch3: Schmallenberg; Coi3: Coimbra; Mon4: Mönninghausen; Tau4: Taubenheide; Kar5: Karlsruhe (Schluttenbach); Ren7: Gladbeck-Rentfort.

The data of Table 4 shows that it is possible to find soils similar to the original EURO-Soils in reality in the field. As expected, common agricultural soils belonging to SIM-Soil class ES3 are most easily found. However, it is a difficult task, since usually the information provided in soil maps is not detailed enough to find samples which fall exactly into the required class. In addition, it was especially difficult to find soils belonging to SIM-Soils ES1 and ES2, since the original EURO-Soils were identified in the Mediterranean region. As mentioned earlier a difference in one of the four classes was allowed in order to keep a natural soil within the respective SIM-Soils class. The Standard soil LUFA 2.2 was classified as ES3 despite the fact that it is a sandy loam and not a “true” loam. In fact the whole approach requires a certain amount of expert knowledge in order to classify natural soil samples. In any case there are many soils which cannot be classified into one of the SIM-Soils defined so far, simply because the number of the original EURO-Soils was restricted in order to cover the most representative European soils and simultaneously keep it practical. Obviously, the OECD artificial soil also stays outside the SIM-Soils approach.

5. Discussion and Outlook

Assuming that the original aims and solutions when defining the EURO-Soils are still valid (independent of the limitations already discussed in chapter 2) it is considered that the SIM-Soils approach is a logical extension. As could be shown in

the last chapter it is possible to find natural soils belonging to the individual classes despite some practical problems. Two of them are worth mentioning:

- The classes have to be re-defined when more practical information with a higher number of natural soils has been investigated. Most problematic is the fact that the individual values can differ when different batches of the same soil from the same site are measured. Speaking more generally, any classification based on fixed trigger values is problematic since in reality a continuum of soils exists. In the long run, the approach presented here has to be replaced by a more flexible system based on multi-variate statistical methods.
- Up to now, the EURO-Soils as well as the SIM-Soils have been defined purely by soil properties. However, since the SIM-Soils are defined to be used as substrates for biological tests the reaction of the main test species has to be taken into consideration when fixing the details of the SIM-Soils classes.

In order to clarify which soil invertebrate test species can survive and reproduce in which EURO-Soils and/or SIM-Soils a two-tiered project was started. In the first step, the behaviour of four species belonging to two important organism groups (*Folsomia candida* and *Hypogastrura assimilis* (Collembola) and *Enchytraeus albidus* and *Enchytraeus luxuriosus* (Enchytraeidae)) in the soils presented in this paper is determined (similar work is underway with other organism groups including earthworms (e.g. Stephenson and Feisthauer 2003). In the second step, the effects of two model chemicals (the herbicide Phenmedipham and the heavy metal copper) will be studied in order to identify the influence of these chemicals on the different test species in the 11 SIM-Soils plus the two artificial and the natural standard soil LUFA 2.2. The results of these two test series will be published in detail elsewhere. However, it can be expected that the organisms due to their different physiology and life-style will react differently to the soil properties and the chemicals (Amorim *et al.* 2004a, 2004b; Jänsch *et al.* 2004).

Actually, the SIM-Soil approach (albeit without applying the name) has already been used once in a project sponsored by the European Union: an ecotoxicological semi-field method with Terrestrial Model Ecosystems (TMEs) was validated by performing tests at four sites in Europe (Knacker *et al.* 2004). The soils to be tested were selected in such a way that they were reminiscent of one of the EURO-Soils in order to facilitate the extrapolation of the results from the test sites to wider areas of Europe.

However, despite the promise of the experiences available and the projects running right now, it is clear that further data concerning the use of SIM-Soils in invertebrate tests are needed in order to clarify in which SIM-Soil(s) which test(s) can be performed in a reliable way. Very probably, some soils cannot be used with the standard test species, e.g. very acid tundra soils. In such cases new tests have to be developed – or at least existing tests have to be modified in a way that, for example, an acidophil oligochaete species can be tested (e.g. the earthworm *Dendrodrilus rubidus* instead of the compost worm *Eisenia fetida* (Rundgren and Nilsson 1997)). The same is probably also true for plant tests: If the growth and reproduction of the most common plant test species is known in different SIM soils (a work which has already been started (Jessen-Hesse *et al.* 2003)), the selection of a test soil will become easier in the future.

When accepting the SIM-Soil approach in general, several detailed questions have to be clarified. In the case of testing individual chemicals it is unlikely that OECD artificial soil will be discharged completely in the future (in any case it would make no sense to exchange the artificial soil by one SIM soil). Instead, artificial soil could be used for testing on a first tier (e.g. comparable to the “base set” in aquatic testing, probably with a reduced amount of organic matter (5 % instead of 10 %?)). In addition, it might serve as an external control in order to secure the quality of the individual test system. SIM-Soils are used in the standard invertebrate tests but the required number, differentiated according to the tiers of the whole test strategy, has to be fixed. Depending on the results of the tests of the first tier or on the area where the test chemical will be applied, additional soils can be requested. For example, the

European Union already acknowledges the existence of different regions within Europe (FOCUS 1996). These regions are mainly defined by climatic data but soil data are given in their description. However, these issues have to be addressed in a process in which all potential stakeholders have to be involved (governmental authorities, industry, universities, standardisation organisations), since besides science clearly legal and economical requirements have to be taken into account.

In addition, SIM-Soils can be used for the assessment of soil quality. Their use as parallel controls to potentially contaminated soil samples, as a dilution substrate for contaminated samples or as a reference soil for monitoring programmes is possible. Due to the "open" definition of SIM-Soils their use is very flexible. In order to keep the whole SIM-Soil approach practical and also to get acceptable results, it is highly recommended to provide sufficient guidance for their use before implementing the SIM-Soils.

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Chapter 3

Identification of the ecological requirements of the most important terrestrial ecotoxicological test species

3. Identification of the ecological requirements of the most important terrestrial ecotoxicological test species

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ABSTRACT

For about 20 years, standardised soil ecotoxicological tests usually rely on the use of an artificial soil substrate (e.g. Organisation for Economic Co-operation and Development; OECD). For both the extrapolation of data obtained in the laboratory to the field situation as well as the biological assessment of contaminated sites this approach alone is not sufficient anymore. For this reason a literature review has been performed to investigate the ecological requirements of the most important terrestrial ecotoxicological test species. The invertebrate species covered were *Eisenia fetida*, *E. andrei* (earthworms), *Enchytraeus albidus*, *E. crypticus* (potworms), *Folsomia candida* (springtails), and *Hypoaspis aculeifer* (predatory mites). The ecological parameters included were pH-value, moisture, temperature, soil (i.e. texture, water-holding capacity, organic matter content, etc.), and food. From the information gathered it may be concluded, for what range of natural soils which of the above mentioned species are suitable. The results indicate that most of these species should be applicable to a wide range of natural soils while for some "extreme" soils (e.g. very acid forest soils) alternative test species will be required. Thus further research is required for identifying such species as well as to fill the gaps of knowledge concerning the ecological requirements of the species investigated here.

Keywords: Collembola, Enchytraeidae, Gamasid mites, Lumbricidae, natural soils

1. Introduction

For about 20 years, standardised ecotoxicological tests for the environmental compartment soil have been available. In most of these methods, in particular those using invertebrates, a so-called artificial soil (= a mixture of sand, clay, peat, calcium carbonate and water) is used as the test substrate (OECD 1984). Chemicals to be assessed are spiked to this substrate in various concentrations and their acute and chronic effects on introduced organisms (earthworms, enchytraeids, collembolans and predatory mites) are measured. Despite the fact that artificial soil will be the substrate of choice for reasons of standardisation and comparability, this approach alone is not sufficient anymore because:

- When assessing the risk of single chemicals (e.g. a pesticide), it is difficult to extrapolate the results gained in an artificial soil tests to the real field situation with its often completely different natural soils;
- In the case of assessing contaminated sites, it is necessary to test natural soil samples directly taken from these sites. In order to distinguish between potential effects of the soil properties themselves and the contaminants, a control soil is needed which preferably is a soil with the same properties as the test soil but without the contamination. In addition, this control soil can also be used as a mixing material to get a dilution series (ISO 2003*b*).

In both cases, the testing of natural soils that can be extremely diverse in terms of texture, pH, and organic matter content might become necessary. Unfortunately, the organisms used so far in ecotoxicological tests have mostly been selected for artificial soil, so it is uncertain to what extent they can be tested in natural soils. Since it is necessary to determine the ecological requirements of these species concerning the most important soil properties, this contribution has two aims:

- Presentation of the results of a literature review on the ecological requirements of the invertebrate species most often used in soil ecotoxicology;
- Identification of knowledge gaps concerning invertebrate testing of natural soils.

2. Lumbricidae

2.1 Introduction

Earthworms are important members of the soil community due to their ability to change or create their habitat through various activities, thus some species are correctly considered to be "ecosystem engineers" (Emmerling *et al.* 2002; Jones *et al.* 1994; Lavelle *et al.* 1997). Their activities lead to an improved soil structure, i.e. stabilisation of soil aggregates, increase of water infiltration (partly by higher water-holding capacity; Edwards and Shipitalo 1998; Syers and Springett 1983; Urbanek and Dolezal 1992), often to the formation of a humic layer close to the soil surface (mainly in forest ecosystems; Doube and Brown 1998) and to an increased yield in orchards or grassland (e.g. Blakemore 1997).

In temperate regions most of the earthworms responsible for the activities described above belong to the family Lumbricidae (class Clitellata, phylum Annelida; Zicsi 1982). In Southern Europe the number of species is very high (from Southern France alone at least 50 species are known (Bouché 1972; Zicsi and Czusdi 1999)). In Central and Northern Europe only about 25 (plus some introduced (= peregrine) species are known in total; Graff 1953), probably due to the detrimental influence of the ice ages: the earthworms had to re-colonize these Northern areas after the natural soil was destroyed by moving glaciers (Støp-Bowitz 1969).

Earthworms have long been used in ecotoxicological testing, but only two species were included in standard ISO and OECD test protocols. These are the closely related *Eisenia fetida* and *Eisenia andrei* whose biology and ecological requirements as well as their role in ecotoxicological testing are presented in the following sections.

2.2 Use of *E. fetida* and *E. andrei* in ecotoxicology

An excellent overview on the use of *E. fetida* and *E. andrei* in ecotoxicology is given by Spurgeon *et al.* (2003). The effects of chemicals on *E. fetida* and *E. andrei* are relatively well-known, because tests with these animals have been required for the

registration of plant protection products (PPPs) for more than two decades (for a brief overview of test methods see Edwards and Bohlen 1996). In effect this activity turned *E. fetida* into a model organism for assessing the effects of chemicals on terrestrial saprotrophic invertebrates (Spurgeon *et al.* 2003). Still most tests are done according to OECD guideline 207, measuring mainly acute effects (ISO 1993; OECD 1984). The results of such tests cannot easily be extrapolated to field situations, since *E. fetida* and *E. andrei* are tested in an artificial soil with a high organic matter content. Still field-relevant species like *Lumbricus terrestris*, *Lumbricus rubellus* or *Aporrectodea longa* are rarely tested (e.g. Mather and Christensen 1994), because they are much more difficult to breed and handle (e.g. have very long generation times) in comparison to compost worms.

However, in newer tests chronic endpoints like reproduction (ISO 1998; OECD 2004) and avoidance behaviour (Hund-Rinke *et al.* 2002*b*; ISO 2004; Stephenson *et al.* 1998) are used. Complementary to these laboratory tests a field study designed for the investigation of the effects of pesticides on the abundance, biomass and species composition of lumbricid earthworms is available (ISO 1999*b*). On both investigation levels (laboratory and field) the importance of behavioural endpoints has been highlighted in recent years. For example, Mather and Christensen (1998) pointed out that after application of the fungicide benomyl concentration-dependent earthworms increasingly migrated to the surface, where they were exposed to unfavourable climatic conditions as well as to predators. This behaviour is also species-specific, leading to changes in the community which are very difficult to predict from laboratory tests (performed usually in small closed vessels) or from "normal" field studies (performed in small plots without barriers).

Finally, some non-standardised approaches for the study of the effects of chemicals on earthworms should be mentioned: the use of several biomarkers has been proposed as "early warning indicators", but the field relevance of these new methods is not really clear so far (for a review see Scott-Fordsmand and Weeks 1998). Despite the fact that a great deal of data concerning the bioaccumulation of chemicals by *E. fetida* and *E. andrei* is available, this data is difficult to compare

since no standardised method is available. However, a proposal has been made to OECD recently (Bruns *et al.* 2001). Advanced evaluation methods may provide a more suitable estimate of the exposure and toxicity of a specific compound, in particular when relating the effect to critical body burdens and not concentrations in soil (Kuperman *et al.* 2003*a*; Lanno and McCarty 1997).

2.3 Short description of the biology of *E. fetida* and *E. andrei*

For a long time *Eisenia fetida* Savigny 1826 and *Eisenia andrei* Bouché 1972 have been regarded as one species due to their similar appearance, ecological requirements, and frequent association. They have a mean length of 60 - 120 mm, a diameter of 3 - 6 mm and a segment number varying from 80 to 120 (Reinecke and Viljoen 1991*b*). André (1963) distinguished two forms (one clearly striped and one uniformly coloured), naming them *var. typica* and *var. unicolor*. He proved those two varieties can interbreed but their hybrid offspring is sterile. Graff (1978) discovered indications hinting at physiological races of *E. fetida*. Bouché (1972) gave them the status of subspecies (*E. fetida fetida* and *E. fetida andrei*) until biochemical methods revealed them to be two truly distinct species (e.g. Jaenike 1982; Øien and Stenersen 1984; Robotti 1983). Hence, large parts of the available information on the biology and ecology of these species apply to both species. For this reason first *E. fetida* s.l. shall be introduced as a representative of both species and following that some known differences between *E. fetida* and *E. andrei* shall be pointed out.

In this context it must be mentioned that compost worms are found worldwide today in many different regions and climatic zones (including the tropics), often forming physiological races (Garcia 2004; Knieriemen 1984; Raquet 1983). If not stated otherwise, all information provided here refers to animals raised under temperate conditions.

E. fetida earned its name based on its defence mechanism: when threatened, e.g. put on the end of a hook, it discharges a noxious yellowy, fetid-smelling fluid (Knight 1989; Monroy *et al.* 2002*a*). It is a peregrine epigeic species that was spread by man

all over the world (Blakemore 2002). Michaelsen (1910) suspected the original range of this species in the Caucasian and the northern edge of the Elbrus Mountains in the near east. Omrani (1973) believed *E. fetida* to have originated as a corticolous species from the mountain forests south of the Caspian Sea. The natural habitat of *E. fetida* is under the bark of dead tree trunks and in highly organic forest soil (Dunger 1964; Lee 1985; Ponge and Delhaye 1995; Ronde 1960; Satchell 1983; Wilcke 1953; Zicsi 1968), but it is rarely found in non-anthropogenic biotopes. It mainly occurs in man-made accumulations of rich organic material such as compost heaps, cattle dung, and sewage sludge, giving *E. fetida* the common name "compost worm". Here it can reach high densities of up to 4000 - 5000 ind./m² (Monroy *et al.* 2002*b*).

E. fetida is considered to be the most prolific earthworm. It is reproductively active throughout the year, and is capable of reproducing itself as an amphimictic hermaphrodite (Hartenstein *et al.* 1979). There is also evidence of uniparental reproduction, mainly in *E. andrei* (Domínguez *et al.* 1997, 2003; see below; Hartenstein *et al.* 1980; Sims and Gerard 1985). *E. fetida* can be reproductively active for more than 500 days (Venter and Reinecke 1988). Watanabe and Tsukamoto (1976) found the peaks of cocoon deposition in spring and autumn. This could be confirmed by Monroy *et al.* (2002*b*) in an outdoor heap of cow manure. The number of mature earthworms and the number of matings remained stabilized throughout the year, except in spring when these numbers significantly increased. The number of juveniles was higher in spring and summer and lower in autumn. Cocoons are mostly deposited close to the surface (upper 6 cm; Reinecke and Viljoen 1991*a*). Cocoon production (11 - about 200 per year and individual), the amount of juveniles per cocoons (1 up to 11), time until maturity (4 weeks up to over one year), cocoon incubation duration and growth depend strongly on temperature and food quality (Boström 1988; Evans and Guild 1948; Graff 1974; Lee 1985; Reinecke and Viljoen 1990; Tomlin and Miller 1980; van Gestel *et al.* 1992; see also below).

2.3.1 Biological differences between *E. fetida* and *E. andrei*

Except for the more uniform pigmentation pattern of *E. andrei* the two species are generally regarded as morphologically undistinguishable. However, also the

distribution pattern of segment number and the location of the clitellar organs (slightly posterior in *E. andrei*) may assist in identification (Omodeo and Rota 2004). Haimi (1990) studied the differences in growth and reproduction of *E. fetida* and *E. andrei* and found both growth and net production of hatchlings to be somewhat higher in *E. andrei*. Reinecke and Viljoen (1991*b*) also found a higher cumulative cocoon production and higher number of hatchlings per cocoon in *E. andrei*. Elvira *et al.* (1996) conducted a comparative study of *E. andrei* and *E. fetida* in different organic residues. They found that *E. andrei* needed less time for clitellum development and cocoon production than *E. fetida*, while the latter had a slightly higher cocoon size, longer hatch period and number of hatchlings per cocoon. When both species were bred together, no negative effects on growth and reproduction were detected. Domínguez *et al.* (2003) observed uniparental reproduction in *E. andrei* and *E. fetida*. The percentage of isolated earthworms that produced cocoons was significantly higher in *E. andrei* (33%) than in *E. fetida* (3.5%). Number of cocoons produced and the number of hatchlings were also significantly greater in *E. andrei* (Table 1).

Table 1. Biological differences between *E. fetida* and *E. andrei.*

Biological Parameter	**E. fetida**	**E. andrei**	**Reference**
Growth	lower	higher	Haimi, 1990
Clitellum development	slower	faster	Elvira *et al.*, 1996
Cocoon production	lower	higher	Reinecke & Viljoen, 1991b
	slower	faster	Elvira *et al.*, 1996
Number of hatchlings per cocoon	lower	higher	Reinecke & Viljoen, 1991b
	higher	lower	Elvira *et al.*, 1996
Net production of hatchlings	lower	higher	Haimi, 1990
Cocoon size	higher	lower	Elvira *et al.*, 1996
Hatch period	higher	lower	Elvira *et al.*, 1996
Uniparental reproduction	lower	higher	Domínguez *et al.*, 2003

2.4 Environmental factors influencing *E. fetida* and *E. andrei*

2.4.1 pH value

Like most lumbricid species, *E. fetida* and *E. andrei* clearly prefer neutral to slightly

acid conditions (Table 2). According to Edwards (1988), *E. fetida* tolerates pH values from 4 to 9, while Kaplan *et al.* (1980) found 100% mortality at pH <5 and >9 for this species. The optimum for weight gain was around pH 7.0. For *E. andrei* van Gestel *et al.* (1992) found the optimum for cocoon production at pH 5 - 6. At an initial pH of ≥ 7 cocoon production was significantly reduced compared to pH 6. Cocoon hatchability was significantly increased at pH 7. The number of juveniles per cocoon at pH ≥ 7.5 and the number of juveniles per worm per week were significantly reduced at pH 4, 6.5, 7.5 and 8 compared to pH 6. On the other hand Edwards and Lofty (1977) and Rivero-Hernandes (1991) found an optimum for *E. fetida* of pH 7 - 8. Unfortunately, the method employed for measuring the pH (KCL, CaCl, H_2O) is not always given in the literature, meaning that these differences could be attributed to differing methods in pH measuring. In standard laboratory tests, a pH of 6.0 ± 0.5 is recommended (ISO 1993; OECD 1984).

Table 2. pH-value – optimum and tolerance of *E. fetida* and *E. andrei*.

Optimum	Tolerance	Parameter	Species	Reference
-	4 – 9	Survival	E. fetida	Edwards, 1988
-	5 – 9	Survival	E. fetida	Kaplan, 1980
7	-	Weight gain	E. fetida	Kaplan, 1980
5 – 6	-	Cocoon production	E. andrei	Van Gestel *et al.*, 1992
7	-	Cocoon hatchability, number of juveniles per worm and week	E. andrei	Van Gestel *et al.*, 1992
7 - 8	-	Production	E. fetida	Rivero-Hernandes, 1991

2.4.2 Temperature

The preferences of *E. fetida* and *E. andrei* concerning temperature have been extensively investigated. Most authors found the overall optimum temperature at 20 - 25°C (Lee 1985; Nakamura 1984; Tomlin and Miller 1980; van Gestel *et al.* 1992). The temperature tolerance of *E. fetida* is quite high (0 - 35°C; Edwards and Bater 1992). However, reproduction ceases at temperatures below about 15°C and above 30°C (Nakamura 1984; van Gestel *et al.* 1992). In general growth increases and length of life-cycle and incubation period of cocoons are shortened with increasing

temperature while cocoon hatchability and number of hatchlings per cocoon are reduced at temperatures above 25°C (Edwards and Bater 1992; Tomlin and Miller 1980; Tsukamoto and Watanabe 1977). Cocoons of *E. fetida* do not survive frost (Holmstrup *et al.* 1990). In the tropics, compost worms are often found (e.g. in the area of Manaus, State of Amazonas, Brazil) to thrive at temperatures of at least 28°C (Garcia 2004). No effects of these high temperatures on juvenile development like abnormalities of the clitellum as reported from Graff and Joschko (1994) at temperatures of >30°C for temperate *Eisenia fetida* have been reported from these places. In standard laboratory tests, room temperature (20 ± 2°C) is required (ISO 1993; OECD 1984).

2.4.3 Moisture

In laboratory experiments in organic substrates the optimum actual moisture level for growth and reproduction of *E. fetida* has been determined to be 85% of dry mass (dm) (Edwards 1988; Edwards and Bater 1992; Kaplan *et al.* 1980). Reinecke and Venter (1987) found the highest cocoon deposition within the narrow range of 65 - 70% moisture, a moisture range preferred by 80% of the juveniles. The study also showed that clitellum development was increased at moisture levels above 64%. In artificial soil tests with *E. andrei* van Gestel *et al.* (1992) found cocoon production significantly lower at a moisture of 35 and 45% compared to 55%. At 85% moisture, cocoon production and fertility was significantly higher. The number of juveniles was not affected by moisture, but the number of juveniles per worm per week was significantly lower at a moisture of 35 and 45%. In standard tests according to OECD or ISO guidelines the recommended moisture level for artificial soil is 40 – 60% of the maximum water-holding capacity (WHCmax), which often (but not always) refers to about 55% actual moisture (ISO 1993; OECD 1984).

2.4.4 Soil

Currently, earthworms are usually tested in OECD artificial soil or a natural standard soil, LUFA 2.2 (Table 3). The former soil has a high percentage of organic matter due to the addition of 10% peat, but the latter is not a poor soil either. In both test substrates the worms can reach high reproduction rates of more than 400 juveniles

per 10 adults, although the validity criterion is just 30 juveniles per 10 adults after 56 days (Hund-Rinke *et al.* 2000; ISO 1998).

Table 3. Physical-chemical properties of OECD artificial soil and LUFA 2.2 standard soil (Garcia 2004). *Data from Junker *et al.* 2004

Parameter	Unit	OECD Artificial Soil	LUFA 2.2
Texture (sand, silt, clay)	%	75.4 – 16.6 – 8*	77 - 17 - 6
pH ($CaCl_2$)		6.1	6.1
P	mg/dm^3	1	324
K		10	49
Na		64	22
Ca	c.molc /dm^3	6.29	10.5
Mg		0.55	0.05
N total	%	0.11	0.19
C org		3.59	2.70
Organic material		6.17	4.64
C/N ratio		32.6	14.2
Fe	mg/dm^3	9	288
Zn		1.30	31.9
Mn		0.41	84
Cu		0.02	8.52
Density	g/cm^3	1.1	1.2
WHCmax	%	56.1	50.0

According to Raquet (1983) and Knieriemen (1984) an optimal carrier substrate for the culturing of *E. fetida* has a high void volume, a good moisture absorbency, a high water-holding capacity, good aeration and structural stability against rotting. Hund-Rinke and Wiechering (2001) investigated the avoidance response of *E. fetida* towards seven uncontaminated reference soils. Soil properties ranged from slightly loamy sands to silty loams, C/N-ratios 7.8 - 45.7 and WHC_{max} 25 - 69%. No significant avoidance behaviour could be observed when comparatively testing all soils.

Feisthauer *et al.* (2004) [1] conducted a reproduction study with *E. andrei* in 12 field-collected reference soils. In the more organic soils, clay loam soils (12.8% OM), a hardwood forest organic soil, and a boreal forest organic soil (20.1% OM; surface soil) reproduction of *E. andrei* was equal to or greater than in the artificial soil (9% OM). Reproduction was significantly lower in the very clayey and sandy boreal forest soils. Surprisingly, the number of juveniles was also significantly lower in another boreal forest organic soil (46.8% OM; surficial soil). Reproduction in the agricultural soils with low to moderate organic matter content (2.9 - 3.5% OM) was either equal to, or lower than, that in the artificial soil.

A comparable study is currently underway in Germany: ten Central European natural soils differing strongly in pH (3.5 – 7.5), organic matter content (2 – 10 %) and texture are being investigated concerning the mortality and reproduction of compost worms (*Eisenia andrei*). The results indicate that there is a strong effect of soil properties on reproduction (not on mortality): The mean number of juveniles differs from 40 to nearly 200 per test vessel (= 10 adults) after 56 days. While this range is not a surprise it is astonishing that even in very acid forest soils (<pH 3.5) the validity criterion of the earthworm reproduction test (in pure artificial (= control) soil > 30 juveniles per test vessel) was met (OECD 2004). In addition, it is worth mentioning that the highest number of juveniles was found in LUFA 2.2 standard soil and OECD artificial soil (Junker *et al.* 2004).

2.4.5 Food

As compost and dung heap dwellers *E. fetida* and *E. andrei* prefer rich dead organic matter such as garden refuse, animal faeces (esp. cattle dung) or sewage sludge (Cluzeau and Fayolle 1989; Elvira *et al.* 1996; Haimi 1990; Kaplan *et al.* 1980, Reinecke and Viljoen 1990). Zicsi (1966) found the greatest euryphagy in *E. fetida* compared to other species. Knieriemen (1984) observed a lower reproduction success of *E. fetida* in nitrogen-rich grass cut compared to different types of domestic animal faeces. According to García *et al.* (1999) the growth and reproduction of *E. andrei* are inversely related: maximum growth and least number of cocoons occurred with a

[1] Feisthauer, N.C., Stephenson, G.L., and Princz, J.I. 2004. *Eisenia andrei* reproduction in field-collected reference soils. (in preparation).

mixture of 3:1 sewage sludge and pine needles, while maximum reproduction rate and highest cocoon production appeared with a mixture of sludge and oak leaves (3:1). Domínguez *et al.* (1997) observed the maximum growth and reproduction rates of *E. andrei* with a mixture of pig slurry with straw and pine needles. Lowest growth occurred in a mixture with oak leaves. *E. fetida* also feeds on protozoa, bacteria and some species of fungi (Flack and Hartenstein 1984). According to Miles (1963) protozoa even constitute an essential part of the diet of *E. fetida*.

2.5 Consequences for the testing of soils

The ecological requirements given here show that *E. fetida* and *E. andrei* are suitable test organisms for the evaluation of most holarctic (i.e. North American and European) soils when sticking to the respective test protocols (Table 4). The only larger groups of soils not suitable to be tested with these species might be strongly acidic forest soils, heavy clay soils and marine soils due to their high salinity (Schaefer 2003). As already stated in the literature (e.g. Edwards and Bohlen 1996), the individual soil properties have to be taken into account when checking a certain natural soil for whether compost worms can be tested in it – a sum parameter like the soil type is surely not sufficient. In addition the fact that, depending on the soil properties, the absolute number of juveniles can vary by a factor of five must be considered, in particular when comparing the test results with the validity criterion defined for artificial soil.

Table 4. Soil ecological preferences and range of *Eisenia fetida* and *E. andrei.*

Soil property	Preference	Range
pH-value	5 - 7	4 - 9
Temperature	20 – 25°C	0 – 35°C (survival) 15 – 30°C (reproduction)
Moisture	65 - 85%	?
Soil	OM: Very high Texture: ?	OM: Wide Texture: Wide
Food	Rich dead organic matter	Wide

3. Enchytraeidae

3.1 Introduction

The enchytraeids belong, like the lumbricids, to the class Oligochaeta and thus to the clade of the Annelida. Among the microdrile annelid worms, they are the closest relatives to the earthworms (Erséus and Källersjö 2004). At present there are about 950 species described worldwide. In Central Europe until 1959 112 species in 16 genera were already recorded (Nielsen and Christensen 1959), but still additional species are being discovered, and a total of 200 – 300 species can be expected for Central Europe alone (Römbke *et al.* 1997); probably 50 – 100 can be classified as common for Central Europe (Didden *et al.* 1997). However, these worms had virtually been ignored in soil biology until the late fifties. Thus, their taxonomy as well as many aspects of their biology have become a subject of study only in recent years.

The enchytraeids belong to the saprophagous mesofauna of the litter layer and the upper mineral soil. Through the feeding activity of enchytraeids the soil assumes a fine-grained crumb structure with an often higher stability than that of the bulk soil. Whether clay-humus complexes also emerge from the digestion process is not completely clarified to date (Didden 1993; Zachariae 1965). Enchytraeids possess a certain digging ability (small compared to most earthworms) and thus may improve the small-scale water- and air-management of the soil. Some species, e.g. *Cognettia sphagnetorum* in acid coniferous forests of Central and Northern Europe, play a key role in processes such as the decomposition of organic matter and nutrient cycling (Laakso and Setälä 1999). Due to their very high biomass and the lack of earthworms at these sites they are considered to be ecosystems engineers there too (Lavelle *et al.* 1997).

Enchytraeids appear with high abundance in different soils with 1 – 30 different species per site worldwide. In terrestrial ecosystems of Central Europe, their average annual abundance lies between 20,000 and 60,000 ind./m², but is subjected to strong seasonal fluctuations (Petersen and Luxton 1982). Their abundance may range from a few thousand up to 100,000 ind./m² and more. Outside of temperate regions, their

mean abundance differs strongly: while in Arctic soils (in particular acid ones) numbers in the same order of magnitude have been reported (e.g. MacLean *et al.* 1977), their numbers are considerably lower in tropical soils (usually less than 10,000 ind./m²; e.g. Römbke and Meller 1999). It seems that in many temperate soils high numbers of abundance and biomass of these two oligochaete groups are reciprocally exclusive (Ponge 2003).

Enchytraeids are hermaphrodites, but some species are able to reproduce via parthenogenesis or self-fertilization. Another reproduction strategy is fragmentation, where an individual autonomously breaks up into several parts, each of which regenerates into a complete individual. However, most species reproduce sexually, by means of egg deposition and fertilization.

A general overview of the ecology of terrestrial enchytraeids is given by Didden (1993). At present there is still little knowledge of the ecological preferences of single enchytraeid species regarding certain soil properties. However, promising efforts to identify the ecological requirements of some Central European enchytraeid species have been made either by local investigations (Graefe and Schmelz 1999; Healy 1980) or by evaluating the data available in the literature (Jänsch and Römbke 2003). From these investigations the genus of *Enchytraeus* (with the exception of *E. norvegicus*) may be generally regarded as neutrophilic with a low tolerance to acidic conditions. Data from literature is also available for *E. albidus* and (less) *E. crypticus*, since these species are frequently used in ecotoxicological testing (Römbke 2003).

3.2 Use of *E. albidus* and *E. crypticus* in ecotoxicology

Despite their important role in many ecosystems the Enchytraeidae had been virtually neglected as test organisms until about 15 years ago (Römbke 2003). Upon discovery of their sensitivity to anthropogenic stress in field studies the Enchytraeid Reproduction Test (ERT), a chronic laboratory test for the testing of chemicals and soil quality assessment utilizing artificial soil, was developed using *E. albidus* (Römbke and Moser 1999, 2002). This test has been internationally ring-tested and is

currently being standardised as a guideline by ASTM (2000), ISO (2003*a*) and OECD (2003). In terms of design it is similar to the earthworm and collembolan reproduction tests (ISO 1998, 1999*a*), having been developed at about the same time.

During its standardisation phase the ERT was successfully modified in terms of test duration, size of the test vessels, amount of test substrate, food and validity criterion "number of juveniles" to also be applicable for soil quality assessment (e.g. Juvonen *et al.* 2000). In addition, the same test can be performed with several species of the genus *Enchytraeus*, such as *E. luxuriosus* (Collado *et al.* 1999). Most notable is the small species *E. crypticus*, which due to its quick development has become very popular in recent years (Achazi *et al.* 1997; Hund-Rinke *et al.* 2002*a*, Kuperman *et al.* 2003*b*). Also additional methods are under development, e.g. a behaviour test for screening purposes using either *E. crypticus* (Achazi *et al.* 1999) or *E. albidus* (Amorim *et al.* 2004*a*) [2] and a bioaccumulation test using *E. albidus* and *E. luxoriosus* (Bruns *et al.* 2001).

3.3 Short description of the biology of *E. albidus* and *E. crypticus*

E. albidus is the best-known and one of the largest species of the genus *Enchytraeus*. It has an average size of about 20 mm. Worldwide it occurs in places with a large amount of organic material, but can rarely be found at crop sites (e.g. Kapusta *et al.* 2003) or in forests, particularly in the middens of the earthworm *Lumbricus terrestris* (e.g. Räty 2004).

Reproduction usually happens sexually (Westheide and Müller 1996) but the possibility of self-fertilization and parthenogenesis has been shown to exist (Gavislov 1935). In LUFA 2.2 soil Achazi (1997) found a reproduction rate for *E. albidus* of 9.3 Ind./6 weeks. Depending on the temperature, the length of the life-cycle can differ between 33 days (18 °C) and 74 days (12 °C) under laboratory conditions (Römbke and Moser 2002). Reynoldson (1943) determined values in the same order of magnitude (ca. 35 – 42 days) at 15 and 20°C, but his individuals were probably mis-identified (according to Albert (1975) they belonged to *E. coronatus*).

[2] Amorim, M.J., Römbke, J., and Soares, A.M.V.M. 2004*a*. Avoidance behaviour of *Enchytraeus albidus*: effects of Benomyl, Carbendazim, Phenmedipham and different soil types. (submitted).

E. crypticus: unfortunately it is not known from where this species originates, since its description is based on individuals from a German compost plant (Römbke 2003). *E. crypticus* has an average size of about 7 mm (Westheide and Müller 1996). It reproduces very rapidly having a generation time of less than 20 days at 20±2°C (Achazi *et al.* 1999). At 21°C on agar plates *E. crypticus* has an average embryological development time of 9.1 days. The average length of time from hatching to maturity is 8.3 days and the average total life span is 85 days. Cocoon production is 0.62 cocoons per day and the mean number of eggs in a cocoon 7.6 with a total range from 1 to 35. Thus the mean number of eggs produced per day is 4.6 (Westheide and Graefe 1992). In LUFA 2.2 soil Achazi (1997) found a reproduction rate of 104 Ind./4 weeks. In comparison to *E. albidus*, the development of *E. crypticus* is clearly quicker, in particular at higher temperatures (Dirven-van Breemen *et al.* 1994; ISO 2003*a*).

3.4 Environmental factors influencing *E. albidus* and *E. crypticus*

Since most ecotoxicological tests with *E. albidus* and *E. crypticus* were conducted with artificial soil (70% sand), in the following section mainly the influences of the different soil properties will be discussed. In Table 6 the effects of actual moisture, pH-value and organic matter content on the reproduction of *E. albidus* and *E. crypticus* are presented (Dirven-van Breemen *et al.* 1994). The data prove that the ecological requirements of both species differ.

3.4.1 pH

E. albidus is acidophobe (optimum: 6.8 - 7.0). In natural soils (e.g. LUFA 2.2) high numbers of juveniles are reached at pH-values of 5.8 - 6.0. *E. crypticus* only avoids strongly acid soils (pH < 4.0). High numbers of juveniles were found at pH-values of 4.8 - 6.5, but the optimum is probably rather at 5.9 - 6.5. Lower numbers were observed at a pH-value < 4.8 and below 4.0 almost none could be found. Higher pH-values up to about 7.7 only had slight effects (Achazi *et al.* 1996; Achazi 1997). Brüggl (1994) found an optimal pH-value for *E. crypticus* of 6.0. Graefe and Schmelz (1999) characterize both *E. albidus* and *E. crypticus* as indicators of slightly acid to slightly alkaline conditions, never to be found in strongly acid soils. In

standard tests (e.g. ISO 2003*a*), a pH of 6.0 ± 0.5 is used.

3.4.2 Temperature

Each of the two species prefers a clearly different temperature ranges: *E. albidus* 15 - 20°C, *E. crypticus* 25 - 30°C (Table 6). The former species has little tolerance against either higher (see also ISO 2003*a*) or lower temperatures (≤ 10°C). Achazi *et al.* (1997) cultivated *E. crypticus* at 20 - 23°C and Brüggl (1994) found an increase in population development of *E. crypticus* at temperatures from 15 - 21°C. This he found to be related to faster embryo and juvenile development. The number of cocoons and eggs per cocoon seemed to be unaffected within this range of temperature. Despite their different requirements, both species are usually tested at 20 ± 2°C (ISO 2003*a*).

Reynoldson (1943) found a temperature dependency of fecundity of *E. albidus* with a maximum at 20°C. The duration of incubation as well as maturation were also positively correlated with temperature. However, the species seems to be rather tolerant towards temperature. Reproduction was possible from 1 – 25.5°C. Below 4.5°C juveniles survived but had not reached maturity after 6 months. Body length does not appear to be temperature related. However, as mentioned before, his species identification is doubtful; so most likely these data refer to *Enchytraeus coronatus* (Albert 1975).

3.4.3 Moisture

E. crypticus prefers less moist soils than *E. albidus* (35 - 55% of dry mass compared to 55 - 65%; Table 6), but both species are able to reproduce at 90% actual moisture. In the standard tests, 40 – 60% of the maximal WHC are required, but actually the "finger probe" allows a better evaluation as to whether the soil has the right moisture. With natural soils a moisture of 60% of the WHCmax is usually sufficient for *E. albidus*, while *E. crypticus* does not appear to have problems with even higher values. At 15% actual moisture both species no longer reproduce (Dirven-van Breemen *et al.* 1994).

3.4.4 Soil

In artificial soil the correlation between the number of juveniles of both species and the organic matter content (at least between 5 and 20%) is weak (Table 6), but with *E. albidus* reproduction decreases slightly if the organic content is as low as 5% (Dirven-van Breemen *et al.* 1994). In fact, also in LUFA 2.2, when a content of organic matter of 4.64% exists, high numbers of juveniles appear. With uncontaminated natural soils reproduction is only inhibited at an organic matter content below 3% (Amorim *et al.* 2004*b* [3]).

Table 5. Main characteristics of the tested soils: pH, OM, C/N, granulometry, CEC and WHC (Römbke and Amorim 2004).

SOIL	pH ($CaCl_2$)	O.M. (%)	C/N	Clay (%)	Silt (%)	Sand (%)	CEC (mval/100g)	WHC (%)
ES1	5.1	2.7	7.7	75	22	3	29.9	62.6
Nat1	6.2	1.7	8.7	33	66	5	40.7	58.4
ES2	7.4	6.4	18.5	23	64	13	28.3	68.5
Hoh2	6.2	12.9	25.0	6	61	33	78.3	73.9
ES3	5.2	6.5	13.3	17	37	46	18.3	42.6
Sch3	5.4	4.1	10.4	23	45	32	68.5	67.4
Coi3	6.7	6.5	17.0	26	60	14	75.8	68.1
ES4	6.5	2.9	9.7	20	76	4	17.5	42.9
Mon4	6.5	2.5	9.7	11	77	12	20.7	53.2
Tau4	6.9	2.9	9.7	17	79	4	61.3	63.1
ES5	3.2	15.9	30.8	6	13	81	32.7	38.7
Eso5	3.2	9.2	29.7	10	12	79	87	100.1
ES7	4.4	11.5	14.2	19	35	46	5	80.6
Ren7	3.8	8.7	11.0	18	40	42	132	121.8
EsoX	6.3	8.9	23.5	31	33	36	-	64
KarX	3.6	10.6	45.9	13	58.5	29	173	71.9
OECD	6.0	9.0	107.5	15	9	76	45.8	58
LUFA 2.2	5.5	3.9	13.5	6	17	77	11.2	55
Total range	3.2 – 7.4	1.7 – 15.9	7.7 – 107.5	6 - 75	9 - 79	3 - 81	5 - 173	38.7 – 121.8

[3] Amorim, M.J., Römbke, J., and Soares, A.M.V.M. 2004*b*. Effect of different soil types on the Enchytraeids *Enchytraeus albidus* and *Enchytraeus luxuriosus* using the herbicide Phenmedipham. (in preparation).

Even a very high proportion of sand (77 - 93%) does not necessarily inhibit reproduction of *E. albidus* in natural soils (Amorim *et al.* 2004*b* [3]; see Table 5 and Fig. 1). However, if high sand content, low organic matter content and a relatively low pH-value (4.8 - 5.6; in one case rather high (7.4)) appear together, the reproduction rate of *E. albidus* is low. This effect was also observed in the case of LUFA standard soils 2.1 and 2.3 (Römbke 1991). Laboratory cultures of *E. albidus* and *E. crypticus* are usually kept in artificial soil, LUFA 2.2 standard soil or gardening soil (or a mixture of these soils), while the latter species can also be successfully kept on agar plates (Achazi *et al.* 1997; 1999; Brüggl 1994; Römbke 1991). In some cases even an organic matter content of 0.3% did not negatively affect *E. crypticus* (Achazi *et al.* 1997).

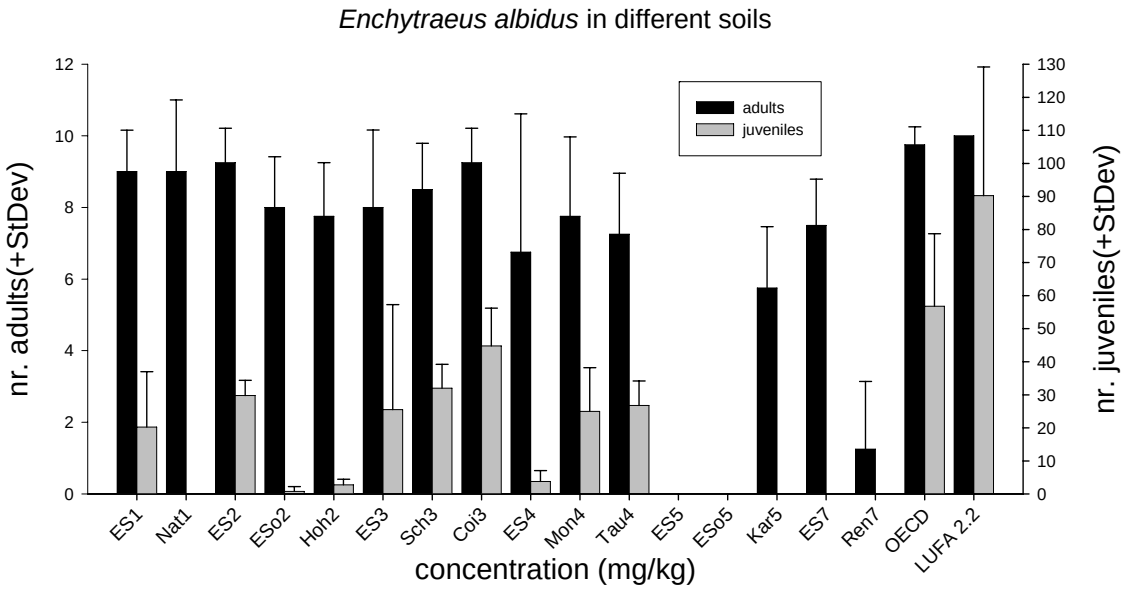

Fig. 1. Results obtained exposing *Enchytraeus albidus* to different soil types (OECD soil, LUFA 2.2, Euro-Soils and similar soils). Graphs show average number of organisms + standard deviation.

Table 6. Effects of pH-value, temperature, actual moisture and organic matter content on the reproduction of *E. albidus* and *E. crypticus* (Dirven-van Breemen *et al.* 1994).

Soil properties	**Number of juveniles per worm and week**	
	E. albidus	E. crypticus
pH-value		
3.2	0.01 ± 0.01	0.64 ± 0.53
3.6	0.01 ± 0.02	3.27 ± 0.51
4.0	0.30 ± 0.42	5.49 ± 2.35
5.3	2.79 ± 1.19	4.78 ± 2.15
6.8	5.75 ± 2.11	9.54 ± 1.55
7.0	7.63 ± 1.14	8.25 ± 0.98
Temperature [°C]		
5	0.00	0.00
10	0.10 ± 0.15	0.00
15	1.35 ± 1.30	0.84 ± 0.28
20	1.23 ± 0.61	1.74 ± 0.83
25	0.00	6.13 ± 1.79
30	0.00	5.85 ± 2.22
Actual moisture [%]		
15	0.0	0.0
35	0.36 ± 0.32	3.80 ± 0.65
55	1.82 ± 0.89	2.65 ± 0.69
65	1.62 ± 0.58	1.43 ± 0.28
90	1.38 ± 0.67	2.11 ± 0.60
Organic matter content [%]		
5	6.4 ± 1.7	4.4 ± 0.6
10	8.8 ± 2.2	4.0 ± 1.1
20	8.2 ± 2.4	4.1 ± 1.4

3.4.5 Food

Laboratory cultures of *E. albidus* and *E. crypticus* are usually fed with ground rolled oats (Achazi *et al.* 1997; Brüggl 1994; ISO 2003*a*), the former also with other organic material like cow-dung, noodles or coffee grounds. In semi-commercial breeding, white-bread soaked in milk has been successfully used (Mr. Benoit, pers. Comm.). Brüggl (1994) found a clear correlation between the amount of food and the population development of *E. crypticus*.

3.5 Consequences for the testing of soils

The data presented here confirm that both *E. albidus* and *E. crypticus*, until now mainly used in single chemical assays with artificial soil or LUFA standard soil 2.2, may also be used for the assessment of soil quality. Due to their ecological requirements relatively moist, usually sandy and slightly acid to alkaline soils (pH 4.8 - >7.0) with an organic matter content of 3% and more could be inhabited by these two species. It seems that *E. crypticus* has somewhat less demanding ecological requirements than *E. albidus* (e.g. in terms of organic matter content or temperature; Table 7). However, not all combinations of these factors are suitable for them. In some cases (e.g. acid soils) clearly other test species with a lower pH-preference (< 4.5) are needed. In any case further studies are necessary in order to determine the preferred range of soil properties in more detail.

Table 7. Soil ecological preferences and range of *Enchytraeus albidus* and *E. crypticus*.

	E.albidus		**E. crypticus**	
Soil property	**Preference**	**Range**	**Preference**	**Range**
pH	6.8 - 7.0	4.8 - 7.4	5.9 - 6.5	3.6 - 7.7
Temperature	15 – 20°C	10 – 20°C	25 – 30°C	15 - ?°C
Moisture	55 -65%	?	35 – 55%	?
Soil	OM: High Texture: ?	OM: 1.5 – >20 Texture: Wide	OM: High Texture: ?	OM: 0.3 – >20 Texture: Wide
Food	Rich dead organic matter	Wide	Rich dead organic matter	Wide

4. Collembola

4.1 Introduction

The Collembola, known as springtails, are the most numerous and widely distributed terrestrial insects. Population densities commonly reach 10^5 m^{-2} in soil and leaf litter layers. Despite their small size (adults: 0.5 – 5 mm long) and low contribution to the total biomass and respiration, they have a very important role as regulators of decomposition processes through microbivory and microfauna predation. They may also contribute to the organic matter breakdown in acidic soils, where earthworms and diplopods are absent (Wiles and Krogh 1998). The Collembola are the best studied soil microarthropods (Crommentuijn 1994; Smit 1997). The species used regularly in ecotoxicological investigations is *Folsomia candida* (Phillips *et al.* 2002), but several other species have been tested, such as *Hypogastrura assimilis* (e.g. Folker-Hansen *et al.* 1996), *Isotoma viridis* or *Orchesella cincta*. Well developed methods have been published for *Isotoma tigrina* (Kiss and Bakony 1992) and *Folsomia fimetaria* (Løkke 1995; Wiles and Krogh 1998). For an overview on the use of collembolan species in soil toxicity tests see Achazi *et al.* (2000).

4.2 Use of *F. candida* in ecotoxicology

Folsomia candida is widely used in ecotoxicology studies, being especially suitable for studying different individual and population parameters in a single experiment, due to its relatively short generation time and parthenogenetic reproduction (Cromentuijn *et al.* 1993). The main reason for this is the fact that a standardized test procedure is already available (ISO 1999*a*).

The effects of chemicals on *F. candida* have not been reviewed in detail recently, but a first overview is given by Hopkin (1997). This might be caused by the fact that there has been a very high number of studies performed since 1972, when Thompson and Gore (1972) investigated the effect of 29 insecticides to *F. candida*. In particular the effects of various pesticides (e.g. Crommentuijn *et al.* 1995; Wiles and Frampton 1996) or metals (Lock and Janssen 2002; Smit and Van Gestel 1998; Van Gestel and Van Diepen 1997; Vijver *et al.* 2001) are well-studied. In addition, the influence of environmental conditions like drought (Holmstrup 1997) on the test results has been

investigated – but not the role of soil properties.

4.3 Short description of the biology of *F. candida*

Folsomia candida Willem 1902 (Collembola: Isotomidae) is a blind, unpigmented euedaphic collembolan that reproduces parthenogenetically. Having reached an age of 20 days (at 20ºC in the laboratory), individuals are about 2 mm long and begin ovoposition. *F. candida* has a well-developed furca (spring organ) and an active running motion. Usually, the species is classified as microsaprophagous, but it can also feed on nematodes (Hopkin 1997). It is widely distributed throughout Europe and although it is not common in most natural soils, it often occurs at humus-rich sites in very high numbers (Wiles and Krogh 1998). Factors that may influence the outcome of parthenogenetically-reproducing collembolans include clonal difference. Crommentuijn *et al.* (1995) found an LC_{50} range of 802 to more than 2024 µg Cd g^{-1} for four different clones of *F. candida*. Also, it is possible to observe that an acceptable range in results is obtained when testing the same clone, e.g., when testing *F. candida* in OECD soil seven times (personal data). Here the average number of juveniles is 807.5 (± 278.2), yielding a maximum number of 1403 and a minimum of 374 juveniles per test vessel, i.e. even under controlled conditions the reproductive output can vary by a factor of about four. So, in any case the number was higher than the validity criterion of 100 juveniles per test vessel in the control after 28 days, as is required by the ISO guideline.

4.4 Environmental factors influencing *F. candida*

4.4.1 pH

Sandifer and Hopkin (1996) tested *F. candida* in the different pH values of 6.0 (the value required by the ISO guideline), 5.0 and 4.5 in a standard laboratory test system with artificial soil. Although no clear relationship between adult survival or juvenile production and soil pH was found, there was an overall decrease in reproduction in the control samples at pH 5.0 and 4.5 in comparison to those at pH 6.0. In a similar experiment, Greenslade and Vaughan (2003) studied an even wider range of pH values, finding an optimum of juvenile numbers at pH-values of 5.38 to 6.62. Interestingly, at lower pH values (down to 3.47) the number decreased to about 50%

of the optimum number, while at higher pH values (7.65 and 8.03) there was a strong decrease down to zero.

In a reproduction experiment performed in a wide range of soil properties (Amorim *et al.* 2004*c* [4]) in which pH ranged from 3.2 to 7.4 (and other soil properties changed simultaneously, since these were mainly natural soils), no significant effect of pH could be identified, although EC_{50}s ranged from 39.2 to 4.4 mg Phenmedipham kg^{-1} soil DW.

4.4.2 Temperature

After having tested different temperatures the overall results suggested that it is possible to conduct the test at temperatures between 10 and 20ºC. However, the test duration has to be adjusted to the temperature: durations of 12, 8 and 4 weeks are recommended at 10, 15 and 20ºC, respectively (Wiles and Krogh 1998). This is confirmed in studies performed by Sandifer and Hopkin (1997) where three different temperatures were tested, 15, 20 and 25ºC; at 25ºC juvenile production was very low in controls and all metal treatments did not allow reliable calculations; at 15ºC the test duration was increased to 6 weeks and it was observed that the level of juvenile production in the control samples was lower than that at 20ºC. Also, the EC_{50} values suggest that at 15ºC, reproduction of *F. candida* is generally more sensitive to all four metals (Cd, Cu, Pb, Zn) than that at 20ºC. Already in 1973, Snider and Butcher stated that 26ºC possibly approaches the upper limit of tolerance for *F. candida*. While some papers (Hutson 1978; Marshall and Kevan 1962; Snider and Butcher 1973) indicate that slight advantages such as increased fecundity and longevity occur at 15ºC, it is not recommended that the test be performed at this relatively low temperature. The results gained at this temperature do not differ significantly from those at 20°C, but a minimum extension of the experimental period of two weeks is required (Sandifer and Hopkin 1997). Again, in a study by Martikainen and Rantaleinen (1999), *Folsomia candida* was exposed to dimethoate at 13, 16 and 19ºC. Incubation temperature per se influenced the collembolans but not the survival

[4] Amorim, M.J., Römbke, J., and Soares, A.M.V.M. 2004*c*. Effect of different soil types on the Collembolans *Folsomia candida* and *Hypogastrura assimilis* using the herbicide Phenmedipham. (in preparation).

of adults. Temperature negatively affected the growth of adults (adults grew faster at lower temperatures) and positively the reproduction (adults delayed their reproduction at lower temperatures and, therefore, had more resources to allocate to growth). The reproduction and toxicity results after 2 weeks at 19ºC were directly comparable to the results after 4 weeks at 13ºC, allowing comparisons of results of toxicity tests conducted at different temperatures within these limits. In the standard test, 20 ± 2°C are required (ISO 1999*a*).

Collembolans can excrete toxic substances from their bodies both by molting and intestine renewal. At high temperatures these processes occur more often and therefore, excretion is more efficient (Joosse and Bucker 1979). It seems that *F. candida* can compensate for the increased activity and hence increased contact with a chemical by increasing excretion. At low temperatures both these functions slow down. Effects of a chemical, therefore last longer at low temperatures because of the lowered activity of the animals (Martikainen and Rantalainen 1999).

4.4.3 Moisture

Based on the literature (Bursell 1970; Edney 1977) it clearly makes no sense to test very dry soils because of the susceptibility of insects to dryness. In the ISO guideline, the moisture content should be 40 to 60% of the total water-holding capacity (WHC). Van Gestel and Van Diepen (1997) have studied the effect of different soil moisture contents: 25, 35, 45 and 55% of dry mass, corresponding to 28, 40, 51 and 63% of the WHC, on the toxicity of cadmium to *Folsomia candida*. No great influence on the bioavailability and toxicity of cadmium occurred. Body weights in the controls were not affected by soil moisture content. With respect to reproduction, apparently the collembolans produced more eggs at lower moisture contents, which also emerged somewhat later than at the high moisture levels. In a reproduction experiment performed in *F. candida* with a wide range of soil properties using natural soils (Amorim *et al.* 2004*c* [5]), there was a significant relationship between juvenile numbers and WHC (negative effect), i.e. confirming

[5] Amorim, M.J., Römbke, J., and Soares, A.M.V.M. 2004*c*. Effect of different soil types on the Collembolans *Folsomia candida* and *Hypogastrura assimilis* using the herbicide Phenmedipham. (in preparation).

the findings of the previous study.

4.4.4 Soil

As mentioned before, the influence of soil properties on the mortality and reproduction of *F. candida* has rarely been investigated. However, recently these endpoints were studied in eleven natural soils (plus OECD artificial soil and the standard LUFA 2.2 soil as controls), covering a wide range of soil properties (Table 5; Amorim *et al.* 2004*c* [6]). With the exception of a negative correlation between adult survival and soil cation exchange capacity (CEC) values there was no influence of soil properties on the collembolans at all (Fig. 2).

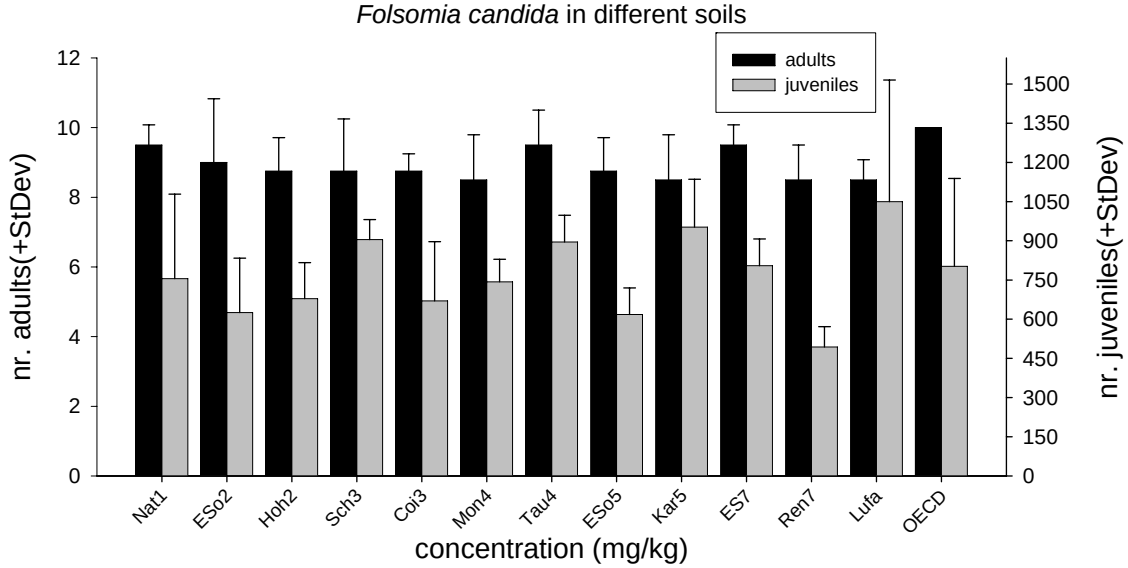

Fig. 2. Results obtained exposing *Folsomia candida* to different soil types (OECD soil, LUFA 2.2, Euro-Soil and similar soils). Graph showing average number + standard deviation.

This observation can be confirmed by results of another study currently underway in Germany (see chapter 2.4.4): The mean number of juveniles differed between 500 and 1000 per test vessel (= 10 adults), thus exceeding the validity criterion of the collembolan reproduction test (in pure artificial (= control) soil > 100 juveniles per

[6] Amorim, M.J., Römbke, J., and Soares, A.M.V.M. 2004*c*. Effect of different soil types on the Collembolans *Folsomia candida* and *Hypogastrura assimilis* using the herbicide Phenmedipham. (in preparation).

test vessel; ISO 1999*a*) by far in all tested soils (Junker *et al.* 2004).

4.4.5 Food

It is assumed that in nature, *F. candida* feeds on fungi (Klironomus *et al.* 1992) although the organism has also been shown to prey on nematodes (Lee and Widen 1996). It is also known that animals fed on pollen performed worse then when fed on yeast (Smit *et al.* 1998; Stam *et al.* 1996). According to the ISO guideline for *Folsomia candida*, food is added in small amounts (2 mg) at test begin and after 14 days. Food consists of dried baker's yeast (*Saccharomyces cerevisae*). Studies have been conducted relative to the effect of a heterogeneous distribution of food. Krogh (1995) studied the importance of special distribution of food by applying it to the surface (common procedure), mixed homogeneously in the whole soil or the top layer, or mixed heterogeneously into the soil. Toxicity decreased significantly when exposure could be avoided in an uncontaminated bottom layer and even more if food was available in this level. But *F. candida* was not able to avoid dimethoate when additionally offered an uncontaminated soil layer. In terms of toxicity, differences were less than one order of magnitude and therefore, for extrapolation purposes the simple basic version may be sufficient. Also, Smit *et al.* (1998) studied the influence of food supply on the toxicity of zinc for *F. candida*, similarly concluding that both zinc stress and food shortage have a major influence on population development.

4.5 Consequences for the testing of soils

The data presented here confirm that *F. candida*, mainly used in single chemical assays with artificial soil or LUFA standard soil 2.2, may also be used for the assessment of soil quality (Table 8). Due to their broad ecological requirements, without specific preferences in terms of organic matter, pH, soils of different textures, can be inhabited by this species.

Table 8. Soil ecological preferences and range of *Folsomia candida*.

Soil property	Preference	Range
pH	6.0	3.2 – 7.65
Temperature	15 – 20°C	10 – 26°C
Moisture	?	wide
Soil	OM: ? Texture: ?	OM: 1 - 11.5 Texture: Wide
Food	Fungi	Wide

5. Gamasid mites

5.1 Introduction

Gamasida (Mesostigmata, Acarina) are the main predators among the soil mesofauna and, therefore, they have a crucial position in the soil food web and contribute significantly to energy and matter turnover (Ruf and Beck 2004). Most species are relatively well-plated and amber-coloured. The highest number of species is found in forests (up to 66), but also in less-worked grassland up to 38 species may be found. Predatory mites are wide spread in many soils (up to 10^4 m^{-2} in both natural and agricultural ecosystems (Krogh and Axelsen 1998)), though they are not as numerous as oribatid mites or collembolans. Densities between 2,300 and 35,000 ind./m² in forests, 2,600 and 20,000 ind./m² in grassland, 2,000 and 120,000 ind./m² in fields and up to 38,000 ind./m² in anthropogenic habitats have been reported. Most species of Gamasida are predators populating the aerated pore-system of soils (Römbke *et al.* 2002). Abundance and community structure are strongly dependent on kind and availability of their prey. The larger surface-dwelling gamasid mites attack small arthropods (collembolans, soft-bodied mites, insect larvae and eggs) and enchytraeids. Smaller deep-litter and soil forms are predominantly nematophagous and are the most important predators of nematodes in many habitats. Some species are fungal feeders. Several genera are considered good bioindicators of habitat and soil condition (The Mite Lab 2003). The ecological role of gamasid mites includes the biocontrol of both pests (nematodes, insect larvae, mites) and beneficials (nematodes, micro-arthropods; Krogh and Axelsen 1998) and thus they are important

species for agricultural sites.

One species of the genus *Hypoaspis*, *H. aculeifer* Canestrini 1883 has recently found its way into the field of laboratory ecotoxicological testing. It has also been widely used in plant protection for the biological pest control of bulb mites (*Rhizoglyphus robini*), fungus gnats (Sciaridae), flies, thrips, mosquitoes or collembolans (Glockemann 1992; Helyer *et al.* 2003; Lesna *et al.* 2000; Lesna and Sabelis 1999). This species is soil-dwelling which makes it especially suitable for the testing of natural soils.

5.2 Use of *H. aculeifer* in ecotoxicology

The history of *H. aculeifer* in laboratory ecotoxicological testing is relatively young. Schlosser and Riepert (1992) were the first to propose a laboratory test protocol with these organisms. Currently two approaches are being further discussed and developed in the course of international standardisation processes:

Krogh and Axelsen (1998) propose a two-species approach, including the collembolan *Folsomia fimetaria* as a prey organism of *H. aculeifer*. This approach aims at a more realistic exposure scenario similar to the exposure through soil and food-chain in the field. In this approach 10 female and 5 male *H. aculeifer* and 100 *F. fimetaria* are introduced per test vessel. After three weeks the endpoints survival, growth, and reproduction are quantified.

Based on experiences in the area of "Non-target arthropod (NTA)" (e.g. with the species *Typhlodromus pyri*), Bakker *et al.* (2003) propose a single-species combined acute and reproduction test. Mortality is assessed after a 14-day exposure period that starts with protonymphs. This is followed by a 7-day mating phase and two oviposition phases (3- and 4-day, respectively) on untreated substrate. Eggs are incubated for 4 to 6 days. After this period the juveniles and non-hatched eggs will be counted. Reproduction endpoint is the number of fertile eggs produced by the surviving females during the 7 days of the oviposition phases.

Currently, an informal research group is discussing a guideline proposal for OECD to be used for the assessment of single chemicals, mainly pesticides. While being based on the experiences collected from the two approaches listed above, the new test shall mainly ensure the continuous exposure of the test animals during the whole test period. Several details like the influence of artificial soil with a reduced content of organic matter (5 instead of 10%) are still under investigation.

5.3 Short description of the biology of *H. aculeifer*

H. aculeifer is a brown-coloured, soil-inhabiting predatory mite. The female is the largest life stage, measuring 0.8 - 0.9 mm and weighing in at 50 - 60 µg (dry weight), and like males (0.55 - 0.65 mm; 10 - 15 µg) possess a pale brown dorsal shield (Helyer *et al.* 2003; Krogh and Axelsen 1998). It usually reproduces sexually but in absence of males also by arrhenotokous (male-producing) parthenogenesis (Krogh and Axelsen 1998; Lesna and Sabelis 1999; Usher and Davis 1983). By selective cannibalism *H. aculeifer* is able to influence the sex ratio in populations (Ruf 1989). The adult female deposits the oval white eggs on the soil surface or in the top layers of compost. After 2 - 6 days (17°C) the colourless six-legged larvae (1 - 3 days at 17°C) hatch and pass through two eight-legged nymphal stages: protonymph (5 - 8 days at 17°C) and deutonymph (6 - 7 days at 17°C; Kevan and Sharma 1964). Development is strongly dependent on temperature (see below). Females reach an average age of 97 days and are able to lay about 100 eggs during their life span (Ruf 1993). Depending on food supply, the females of *H. aculeifer* are able to actively choose their mates. Mate choice varies with diet and is tuned to incorporate 'good genes' in the offspring, that is, genes that promote the population growth rate of the offspring on the same diet as that experienced by the parents (Lesna and Sabelis 1999). The species is peregrine and lives in grassland, humus-rich field and forest soils, moss and decaying vegetation (Krogh and Axelsen 1998; Ruf 1993). Karg (1961*a*, 1961*b*) investigated the annual population dynamics of *H. aculeifer* in Eastern Germany. Females are generally more frequent than males. The maximum abundance was found in August and September. Between November and March almost no animals were caught. Between May and July nymphs seem to be more frequent than adults. The female was concluded to represent the hibernation-stage.

5.4 Environmental factors influencing *H. aculeifer*

5.4.1 pH

There is virtually no information available on the pH preferences or tolerances of *H. aculeifer*. From the majority of its habitats it might be concluded that it has a preference for neutral soils. Also those soils recommended for ecotoxicological testing have a pH value around 6.0 (Krogh and Axelsen 1998). However, since the existence of this species has also been reported in coniferous forests of Southern Finland (Huhta *et al.* 1986) it might be concluded that there is also tolerance for lower pH-values.

5.4.2 Temperature

The life-cycle of *H. aculeifer* depends strongly on temperature. Temperatures below 11°C cause inactivity and a cessation in egg hatch, whereas activity remains high at up to 30°C (Helyer *et al.* 2003). Commercial suppliers recommend an optimal application temperature of 18 - 25°C and critical values of <15 and >35°C. Short-term (1 - 2 days) storage of distribution vessels is recommended at 8 - 18°C (Biobest Biological Systems 2003*a*; Planet Natural 2003; Sautter and Stepper 2003). At 20°C *H. aculeifer* reaches maturity after about 18 days (Helyer *et al.* 2003). The dependence of the development rate is estimated to be practically identical with the bulb mite *Rhizoglyphus robini* (Biobest Biological Systems 2003*a*): At 16°C, it takes ca. 40 days to develop from egg to egg and only 12 days at 27°C (Biobest Biological Systems 2003*b*). Chi (1981) investigated the longevity and reproduction rate at different temperatures. The mean longevity of one female was 194 days at 15°C, 45 days at 22°C and 23 days at 28°C. At these temperatures the female laid 0.42, 1.93 and 2.3 eggs/day, respectively. Kevan and Sharma (1964) observed the development of *H. aculeifer* in relation to temperature. From 11 to 26°C incubation period shortened from 10 to 1 days and duration of larva, protonymph and deutonymph stadium from 4.4 to 1.1 days, 11.4 to 6.3 days and 16.9 to 2.3 days, respectively. Total time to maturity shortened from 43.6 to 11.7 days. Corresponding results were found by Lobbes and Schotten (1980; see Table 9). In testing procedures temperatures of 20°C (Krogh and Axelsen 1998) and 25°C (Bakker *et al.* 2003) are recommended.

Table 9. Cumulative time of development of *Hypoaspis aculeifer* at different temperatures, in d ± SD (Table according to Lobbes and Schotten 1980).

Temp. [°C]	Egg	N	Larva	N	Protonymph	N	Deutonymph	N
15.0	9.9 ± 1.6	54	13.1 ± 1.6	59	21.0 ± 1.4	33	35.8 ± 2.5	29
18.5	7.3 ± 1.3	40	9.6 ± 1.3	48	14.6 ± 1.3	39	27.6 ± 1.4	37
22.5	5.2 ± 1.0	101	7.0 ± 1.3	97	9.9 ± 1.3	128	13.1 ± 1.3	69
24.5	4.3 ± 1.3	125	6.2 ± 0.8	125	9.8 ± 1.1	55	12.5 ± 1.3	69
27.5	3.9 ± 1.0	50	5.0 ± 1.0	91	8.2 ± 1.0	70	11.5 ± 1.3	56

5.4.3 Moisture

The moisture preferences of *H. aculeifer* are not yet adequately investigated. Commercial distributors describe *H. aculeifer* to prefer moist potting compost and other moist spots (Biobest Biological Systems 2003*a*), as well as relatively moist ground (Planet Natural 2003). Slightly moist and well-aerated substrate is considered optimal, but muddy soil should be avoided (Sautter and Stepper 2003). In proposed testing protocols a substrate moisture of 50% of WHC is recommended (Bakker *et al.* 2003; Krogh and Axelsen 1998). With the proposed soils LUFA 2.1, LUFA 2.2 and OECD artificial soil this corresponds to absolute moisture of 20 – 30%.

5.4.4 Soil

H. aculeifer seems to be rather indifferent concerning soil, as long as there is sufficient moisture and prey. It occurs in almost every kind of soil (Glockemann 1992; Ignatowicz 1974), is easily cultured on plaster of Paris (Ruf 1993) and is commercially distributed in vermiculite, e.g. mixed with peat and containing flour mites for food (Biobest Biological Systems 2003*a*). For ecotoxicological testing standard soils LUFA 2.1, LUFA 2.2 or OECD artificial soil is recommended (Bakker *et al.* 2003; Krogh and Axelsen 1998).

5.4.5 Food

H. aculeifer is a polyphagous predator and preys on fungivorous and herbivorous mites, insect eggs and larvae (beetles, flies, thrips and collembolans), enchytraeid worms and nematodes (Bakker *et al.* 2003; Ignatowicz 1974, Lesna and Sabelis

1999; Sardar 1980, Sardar and Murphy 1987; Wiethoff *et al.* 2003). The species is attracted to the food of its fungivorous prey and thus relies on stimuli that lead to an area where the likelihood of encountering prey is high (Hall and Hedlund 1999). When a mite captures its prey it inserts its saw-like mouth parts which slice the internal tissues (Helyer *et al.* 2003). Similar to spiders, they inject a digestive liquid into the prey, and then suck up the dissolved tissue, leaving a shrivelled cadaver (Helyer *et al.* 2003; The Mite Lab 2003). *H. aculeifer* has a high tolerance to starvation, as it can survive for 6 - 8 weeks in the absence of food, although water is required (Helyer *et al.* 2003). There is also cannibalistic behaviour towards eggs and especially of females towards males (Ragusa and Zedan 1988; Ruf 1995). In laboratory cultures the mold mite *Tyrophagus putrescentiae* proved to be a highly suitable food source (Barker 1968; Lobbes and Schotten 1980; Ragusa *et al.* 1986; Sardar 1980; Sardar and Murphy 1987). Apparently *H. aculeifer* is also able to survive on plant material, but reproduction is considerably higher when animal food is presented (Lobbes and Schotten 1980; Ragusa *et al.* 1986).

5.5 Consequences for the testing of soils

From the information gathered here it can be concluded that *H. aculeifer* is a suitable test species for the testing of most soils common in temperate regions (Table 10). For some parameters, i.e. pH-value and moisture, the range of tolerance is not clear to date. In soil testing soil moisture will be adjusted to a specific value, while in the testing of natural soils the pH-value may not be changed. However, in Central Europe most soils subjected to a soil quality assessment will be within the neutral pH-range, since these will usually resemble agricultural and grassland sites. As for whether *H. aculeifer* is also applicable for the testing of e.g. acid forest soils, further investigations on the ecological range of this species are required.

Table 10. Soil ecological preferences and range of *Hypoaspis aculeifer*.

Soil property	Preference	Range
pH-value	6 (?)	?
Temperature	18 – 25°C	11 – 35°C
Moisture	20 – 30% (?)	?
Soil	OM: None Texture: None	OM: Wide Texture: Wide
Food	Tyrophagus putrescentiae	Wide (predatory)

6. Discussion and recommendations

6.1 Individual organism groups

The data presented in this review lead to the following conclusions in respect to the use of standard species in tests with natural soils (Fig. 3).

Earthworms:

- The compost worm species *Eisenia fetida* and *Eisenia andrei* are suitable for many soils of temperate regions – in fact more than usually assumed in the literature;
- However, very acid (pH < 3.5) or very basic (pH > 7.5) soils are not suitable for compost worms. In addition, soils with a very low content of organic matter, a high clay content or those containing salts clearly avoided. Further research is needed in order to define more clearly the influence of the individual soil properties and their interactions;
- Even under tropical conditions some temperature-adapted strains of *E. fetida* can be used in standard tests (Garcia 2004), but no race adapted to cold environments is known;
- For certain questions other species have to be considered in the future, despite the fact that there is no other species so easy to test that it could replace the compost worm. For example, positive experiences are available such as:
  - *Dendrodrilus rubidus*: this species could be tested in acid soils and/or moder-like litter layers (Rundgren and Nilsson 1997);
  - *Lumbricus rubellus*: this large-bodied lumbricid is very suitable for

bioaccumulation studies (Bruns *et al.* 2002);

- *Lumbricus terrestris*: for especial interest in behavioural effects of chemicals on anecic species (i.e. ecosystem engineers) avoidance tests could be performed with this ecologically extremely important species (Stephenson *et al.* 1998);
- *Aporrectodea caliginosa*: due to the fact that many pesticides are used at crop sites, this most common and quite sensitive species dwelling in mineral soil should be used (Booth *et al.* 2000);
- *Perionyx excavatus*: this peregrine species, which originally stems from tropical South-East Asia, could become an alternative to tropical *Eisenia fetida*;
- Generally speaking: the dominant species at a given site with a potentially contaminated soil should be considered for a site-specific assessment, as long as it can be tested in a way similar to standard species and following the same requirements of quality assurance (except that the test organisms come from a long-term laboratory culture; instead the F1 generation of field catches is used).

However, one must be aware that these are always special cases that are not easy to interpret, since usually individuals without a known history are used. In addition, unfortunately comparable data from other laboratories is seldom available.

Enchytraeids:

- The potworm species *Enchytraeus albidus* and *E. crypticus* are suitable for many soils of temperate regions. In general, the ranges are broader for *Enchytraeus crypticus* than for *E. albidus*, for example in terms of pH or organic matter. Further research is needed, in particular concerning the tolerances and preferences to different texture classes of *E. crypticus* – a species whose origin and natural habitat are not known;
- However, for the testing of some soils other species may have to be considered:
  - *Cognettia sphagnetorum*: this species is widespread in Central and Northern European forest soil and has the most pronounced acidophily

of all ecologically characterized Central European species (Graefe and Schmelz 1999; Jänsch and Römbke 2003). It has already been cultured in the laboratory and used in ecotoxicological testing (Rundgren and Augustsson 1998). *C. sphagnetorum* would be especially suitable for the testing of acid soils;

- *Enchytraeus luxuriosus*: this recently described species belongs to the *E. buchholzi/christenseni* – complex, and is smaller and reproduces faster than *E. albidus*. It has successfully been mass-cultured and is recommended as an optional species for the Enchytraeid Reproduction Test (ERT) and in bioaccumulation studies (Amorim *et al.* 2002*a*, 2002*b*; Bruns *et al.* 2001; Collado *et al.* 1999 (as *E. "buchholzi"*)). Results from Amorim *et al.* (2004*b*) [7] show some differences in terms of sensitivity to different soils in comparison to *E. albidus*, but both react similarly negatively to low pH values (ca <5);
- *Enchytraeus norvegicus*: within the genus *Enchytraeus* this is probably the species with the widest ecological spectrum, tolerating a broader range of pH and habitats (Jänsch and Römbke 2003). However, *E. norvegicus* has not yet been used in laboratory ecotoxicological testing and it is not clear if it can be maintained in mass culture.

Collembolans:

- The test species *Folsomia candida* is suitable for all soils tested so far. Further research is recommended in order to determine the influence of different clones on reproductive success and/or the sensitivity towards soil properties;
- However, for certain purposes other species have to be considered in the future, ones that are easy to test and could complement *F. candida* in terms of sensitivity and ecological relevance. For example, important issues to take into account are:

[7] Amorim, M.J., Römbke, J., and Soares, A.M.V.M. 2004*b*. Effect of different soil types on the Enchytraeids *Enchytraeus albidus* and *Enchytraeus luxuriosus* using the herbicide Phenmedipham. (in preparation).

- *Folsomia fimetaria* (Wiles and Krogh 1998) or *Hypogastrura assimilis* (Folker-Hansen *et al.* 1996): These sexually reproducing species could be used to study the effects of chemicals which might affect the reproductive behaviour;
- *Hypogastrura assimilis*: This species seems to be more sensitive to soil properties according to reproduction experiments (Amorim *et al.* 2004*c* [8]). For example, a pH value < 3.8 inhibited reproduction completely;
- *Sinella communis*: Due to local requirements other species might be tested for regional purposes, e.g. this acidophilic one for Australia (Greenslade and Vaughan 2003).

Predatory mites:

- The test species *Hypoaspis aculeifer* is suitable for all soils tested so far;
- However, further investigations are needed to illuminate the ecological range of this species and thus to clarify the suitability of this test species for the testing of "extreme" soils, e.g. forest soils with a very low pH-value. An alternative species that might compensate for gaps in the applicability of *H. aculeifer* is currently not available. Other mesostigmatid species like *Typhlodromus pyri*, which has also been used for the ecotoxicological assessment of pesticides, are leaf-dwelling and thus hardly relevant for soil testing (Bakker *et al.* 2003).

[8] Amorim, M.J., Römbke, J., and Soares, A.M.V.M. 2004*c*. Effect of different soil types on the Collembolans *Folsomia candida* and *Hypogastrura assimilis* using the herbicide Phenmedipham. (in preparation).

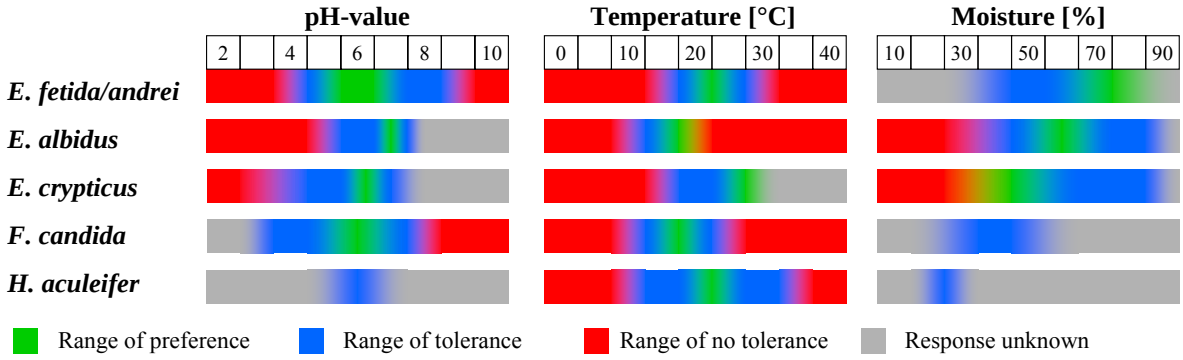

Figure 3. Ecological ranges of common terrestrial ecotoxicological test species regarding pH-value, temperature and moisture.

6.2 General considerations

Summarising the experiences outlined here it becomes clear that standard test species, so far tested mainly in OECD artificial soil or the natural standard soils LUFA 2.2 and 2.1, can also be tested in many natural soils. However, some constraints have to be mentioned:

- Soil arthropods (collembolans, mites) are, as can be expected, less sensitive towards soil properties then soft-bodied oligochaete worms;
- In many cases, research is needed in order to define more clearly the ecological requirements of the standard test species (maybe even for races or clones);
- In addition, research is needed to provide alternative test species necessary to cover certain soils (e.g. sandy acid soils). While such soils have often been classified as “extremes” and thus are often neglected, one should not forget that such soils are very common in, for instance, Scandinavia or Northern Canada. In fact, the same statement is true for most non-temperate and/or non-agricultural soils world wide.

However, due to the growing legal requirements in the field of the ecotoxicological assessment of contaminated soils with biological methods, such research has already started. Some examples (without being exhaustive) include:

- In a ringtest sponsored by the German Federal Environmental Foundation (DBU), it could be shown that earthworms (*E. fetida*), collembolans (*F.*

candida) and enchytraeids (*E. crypticus*) can successfully be used for the assessment of contaminated soils (Hund-Rinke *et al.* 2002*a*);

- The German Ministry for Education and Research (BMBF) is currently sponsoring a project in order to improve the routine implementation of these ecotoxicological methods into site assessment (Jessen-Hesse *et al.* 2003);
- In the context of evaluating the environmental risk of contaminants like mineral oil, the Canadian government is considering the use of native species in order to improve the testing of boreal sites (i.e. often sandy, very acid soils; EC 2004).
- Other countries are boosting the research in the area of ecotoxicological testing with natural soils too: for example, in the United Kingdom a very informative and critical survey of test methods has recently been conducted (Spurgeon *et al.* 2002). In The Netherlands, the interaction between test species, soil properties and certain chemicals has been investigated (e.g. the effects of heavy metals in enchytraeids (Posthuma *et al.* 1998; Peijnenburg et al 1999).
- Last but not least, the whole issue has also been addressed from a pedological point of view. Since it will be impossible to test all occurring soils, it has been proposed to classify the natural soils into a limited number of classes (e.g. according to the criteria set for a selection of soils within the European Union, the so-called EURO-Soils; Kuhnt and Muntau 1992; Løkke *et al.* 2002). The reaction of test species has then only to be tested for these classes, since natural soils (many but probably not all) could be categorized into them appropriately (Römbke and Amorim 2004).

7. Summary

For about 20 years, standardised ecotoxicological tests with invertebrates for the environmental compartment soil have been available. In most of these methods a so-called artificial soil or, less often, a standard natural soil (LUFA 2.2 or 2.1) is used as the test substrate. However, this approach is not sufficient anymore because the results of tests with single chemicals are difficult to extrapolate to the real field

situation with its often completely different natural soils. In the case of assessing contaminated sites, it is necessary to distinguish between potential effects of the soil properties themselves and the contaminants. For these reasons, a literature review has been performed in order to determine the ecological requirements of the standard test species *Eisenia fetida*, *E. andrei* (earthworms), *Folsomia candida* (collembolans), *Enchytraeus albidus*, *E. crypticus* (enchytraeids) and *Hypoaspis aculeifer* (predatory mites) concerning the most important soil properties (pH-value, moisture, temperature, texture, water-holding capacity, organic matter content).

The results of the review indicate that these standard test species can be tested in nearly all (collembolans, mites) or many (earthworms, enchytraeids) natural soils. For *Eisenia fetida* and *E. andrei* strongly acid (e.g. forest) soils or soils with an extremely high sand content or low organic matter content will be problematic. The same applies to *Enchytraeus albidus* and *E. crypticus*, with the latter having a wider range of tolerance concerning pH-value and organic matter content. Both *Folsomia candida* and *Hypoaspis aculeifer* seem to be tolerant to virtually all kinds of natural soils. However, it also became clear that further research is needed in order to define more clearly the ecological requirements of the standard test species (maybe even for races or clones, e.g. for *Folsomia candida*). This is especially true for the upper pH-range for *Enchytraeus albidus* and *E. crypticus*, the pH-tolerances and preferences of *Hypoaspis aculeifer* and the moisture tolerances and preferences of *H. aculeifer* and *Folsomia candida*. In addition, research is needed to provide suitable alternative test species necessary to cover the above mentioned soils often referred to as “extreme” (e.g. sandy acid soils). Due to the growing legal requirements in the field of the ecotoxicological assessment of contaminated soils with biological methods, such research has already started (e.g. in Germany, Canada, The Netherlands and the United Kingdom). Since it will be impossible to test all natural soils the idea is plausible to classify them in a limited number of classes (e.g. according to the criteria set for the so-called EURO-Soils). The reaction of test species has then only to be tested for these classes.

Thus, putting available experience and current efforts together, the routine study of standard invertebrate test species in natural soils, either for the risk assessment of single chemicals or of contaminated soils, is possible from a methodological point of

view.

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Chapter 4

Avoidance behaviour of *Enchytraeus albidus*: effects of Benomyl, Carbendazim, Phenmedipham and different soil types

4. Avoidance behaviour of Enchytraeus albidus: effects of Benomyl, Carbendazim, Phenmedipham and different soil types

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ABSTRACT

Enchytraeids are typical inhabitants of many soils, contributing to vital processes of this environmental compartment. Indirectly they are involved in regulating the degradation of organic matter, as well as improving the pore structure of the soil. Due to their behaviour, are able to avoid unfavourable environmental conditions. Avoidance tests with enchytraeids, initially developed with earthworms by several authors, are quick and easy to perform. With these tests a first assessment of the toxicity of a (contaminated or spiked) soil is possible in just 48 hours by using the reaction of the enchytraeids as measurement endpoint. In this period of time the organisms can choose between the control soil and the other soil (a contaminated or spiked or another soil with different physico-chemical properties). In the tests reported here, the enchytraeids were exposed to control soils spiked with the fungicides Benomyl and Carbendazim and the herbicide Phenmedipham. Several chemical concentrations were tested in order to evaluate the avoidance behaviour to toxic substances. In fact, often these short-term screening tests gave results in a range similar to the acute test results but higher than in chronic tests. Further tests are needed to decide whether the results gained in this study can be extrapolated to other chemicals. It is proposed to standardize the Enchytraeid Avoidance Test as it is currently done for the Earthworm Avoidance Test by the International Standard Organization (ISO).

Keywords: Avoidance laboratory tests, enchytraeids, pesticides, field soils, ecotoxicology;

1. Introduction

Avoidance tests are based on the fact that oligochaete worms possess chemoreceptors highly sensitive to chemicals in their environment (Edwards and Bohlen, 1996; Römbke and Schmidt, 1999). The first attempts of using this kind of tests were performed with *Eisenia fetida* and *Lumbricus terrestris* (Yeardley *et al.*, 1996; Slimak, 1997; Stephenson *et al.*, 1997; Hund, 1998). The avoidance behaviour is an ecologically relevant endpoint since it is directly related with the energy budget of the individual worms and indirectly (i.e. via the moving and burrowing activity) with the soil structure. The major advantage of avoidance tests with soil organisms is the short duration (48h) in comparison to existing acute or reproduction tests (e.g. earthworms: 8 weeks (ISO, 1998) or enchytraeids: 6 weeks (ISO 2003)). Moreover, the test is less laborious (e.g. no counting of juveniles is needed) then other tests. For these reasons, an avoidance test is a promising candidate for a short-term screening test which is so far missing in the battery of test guidelines with terrestrial invertebrates.

The avoidance behaviour of earthworms in relation to soil properties (e.g. Hund-Rinke and Wiechering, 2001) and towards chemicals is relatively well studied, e.g. Slimak (1997) analysed the effect of ten pesticides with different chemical structures in *Lumbricus terrestris*. Thus, the earthworm avoidance test is already on the way to become an ISO guideline (ISO 2004). Much less work on avoidance behaviour has been done with enchytraeids and most of this work has not been adequately published so far (Römbke *et al.*, 2000). However, it is clear that an enchytraeid avoidance test needs further development.

The objectives of this study were to understand whether Enchytraeus albidus can avoid various chemical substances and to study the influence of certain soil properties on the avoidance behaviour associated with the presence of chemicals. Standard soils, i.e. OECD (Organization for Economic Cooperation and Development) artificial soil (OECD, 1984) and LUFA 2.2 soil, were used as reference material in comparison to other natural soils (selected according to the EURO-soil concept (Gawlik and Muntau, 1999) whilst looking for the best reference soil. In addition, the relationship between avoidance behavioural effects and acute (i.e. mortality) as well as chronic (i.e. reproduction) effects were studied based on the

respective LC50 and EC50 values (ISO 2003). The main outcome of these studies is an assessment of the current situation concerning enchytraeid behaviour testing.

2. Materials and Methods

Test Organism

The test organism used belongs to the species *Enchytraeus albidus* Henle, 1837. Individuals were maintained in laboratory cultures, kept in the dark at 20ºC and fed once a week with finely ground and autoclaved rolled oats (Cimarrom, Portugal). Details of the culturing process are given in Römbke and Moser (2002).

Soils

The artificial OECD soil (OECD, 1984) and the natural standard soil LUFA 2.2 (Løkke and van Gestel 1998) were used as reference soils for comparison purposes. Other field soils were selected according to the Euro-Soil concept (Gawlik and Muntau, 1999). At first, it was planned to use the original Euro-Soils in the tests. However, due to the scarcity of the original material this was only possible in the case of ES7 from Austria. Therefore, in two cases soil from the original Euro-Soil site was sampled (ESo 2 from Greece, and ESo 5 from Germany). The remaining soils were selected in a way that they had properties similar to the original Euro-Soils, but were found elsewhere. In these cases the codes represent the first 3 letters of the sampling place followed by a number indicating the respective Euro-Soil (Nat 1 = Natzungen, Hoh 2 = Hohenlimburg, Coi2 = Coimbra, Sch3 = Schmallenberg, Mon 4 = Mönninghausen, Tau 4 = Taubenheide, Kar x = Karlsruhe (Schluttenbach), Ren 7 = Gladbeck-Rentfort). With the exception of the soil from Greece and Portugal (Coimbra), all soils were sampled in Germany. The properties of these soils are given in Table 1.

Table 1: Pedological properties of the tested soils: pH, OM (Organic Matter), C/N (Carbon/Nitrogen), grain size distribution (clay, silt, sand), CEC (Cation Exchange Capacity) and WHC (Water Holding Capacity).

SOIL	pH (CaCl2)	O.M. (%)	C/N	Clay (%)	Silt (%)	Sand (%)	CEC (mval/100g)	WHC (%)
ES1	5.1	2.7	7.7	75	22	3	29.9	62.6
Nat1	6.2	1.7	8.7	33	66	5	40.7	58.4
ES2	7.4	6.4	18.5	23	64	13	28.3	68.5
Hoh2	6.2	12.9	25.0	6	61	33	78.3*	73.9
ES3	5.2	6.5	13.3	17	37	46	18.3	42.6
Sch3	5.4	4.1	10.4	23	45	32	68.5	67.4
Coi3	6.7	6.5	17.0	26	60	14	75.8	68.1
ES4	6.5	2.9	9.7	20	76	4	17.5	42.9
Mon4	6.5	2.5	9.7	11	77	12	20.7	53.2
Tau4	6.9	2.9	9.7	17	79	4	61.3	63.1
ES5	3.2	15.9	30.8	6	13	81	32.7	38.7
Eso5	3.2	9.2	29.7	10	12	79	87*	100.1
ES7	4.4	11.5	14.2	19	35	46	5	80.6
Ren7	3.8	8.7	11.0	18	40	42	132*	121.8
ESo2	6.3	8.9	23.5	31	33	36	-	64
Kar x	3.6	10.6	45.9	13	58.5	29	173*	71.9
OECD artificial	6.0	9.0	107.5	15	9	76	45.8*	58
LUFA 2.2	5.5	3.9	13.5	6	17	77	11.2	55

*means that the CEC was evaluated with uncertainty.

Chemical application

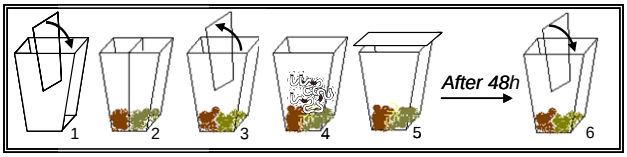
The contamination of all test substrates was done by mixing aqueous solutions of the chemicals into the pre-moistened soils, each test concentration into the whole batch of soil. After homogeneous mixing, the soil was introduced into the test vessels. Test

items were the fungicides Benomyl (Benlate, Sólo de DuPont, 50%) and Carbendazim (Derosal, AgrEvo, 360g/l), and the herbicide Phenmedipham (Betosip, Stähler Agrochemie, 157g/l). All concentrations are given as active ingredient (a.i.) per kg soil (dry weight).

Experimental procedure

The experimental material and procedure used in these experiments is a simple one and was adapted from the existing earthworm test to the size of these small worms: plastic boxes (8x5x10cm) and a movable wall which divides the box in two halves (Fig. 1 and Fig. 2). Previous to the introduction of the soils (25gr in each side), the wall is placed at the centre of the box and the control soil is introduced in one side of the vessel and the test soil on the other. After this, the wall is gently removed and ten adult enchytraeid worms are left on the contact line of the soils. The box is covered with a lid (containing small holes) and the test is running for 48 hours at 20ºC and a photoperiod of 16:8h. At the end of the test the movable wall is replaced in the centre and each side of the box is independently searched for worms.

Scheme 1: Schematic representation of the experimental procedures of enchytraeid avoidance test.



1) Introduction of the movable wall in the centre of the test vessel; 2) Introduction of the soils to be tested; 3) Movable wall is removed; 4) Placement of the enchytraeid worms in the centre of the soils; 5) Covering the test vessel with a lid (perforated); 6) Reintroduction of the wall to separate the soils and counting of the organisms present in each side.

Several experiments were performed and are listed in Table 2.

Table 2: Summary of the experiments performed in the present study.

Experiment	Reference soil	Test Situation	Toxic Concentrations (mg a.i./kg)
1.1	LUFA 2.2	10 Different soils	Control
1.2	OECD	10 Different soils	Control
2.1	LUFA 2.2	Benomyl	0.32 – 1 – 3.2 – 10 – 32
2.2	OECD	Benomyl	0.32 – 1 – 3.2 – 10 – 32
3.1	LUFA 2.2	Carbendazim	0.32 – 1 – 3.2 – 10 – 32
3.2	OECD	Carbendazim	0.32 – 1 – 3.2 – 10 – 32
4.1	LUFA 2.2	Phenmedipham	1 – 3.2 – 10 – 32 – 100
4.2	OECD	Phenmedipham	1 – 3.2 – 10 – 32 – 100
5.1	Nat1	Phenmedipham	1 – 3.2 – 10 – 32 – 100
5.2	Hoh2	Phenmedipham	1 – 3.2 – 10 – 32 – 100
5.3	ES7	Phenmedipham	1 – 3.2 – 10 – 32 – 100

Statistics

Calculations were performed using the statistical software package SPSS 12.0. The avoidance effect expresses the percentage of affected worms (i.e. those which avoided the treated part of the test vessel), and was used as an endpoint. EC50s were calculated as Probit regression, assuming that in the control 50% of the worms are in each side of the vessel (no effect).

Results are presented in graphs in terms of average of net response (NR) expressed as percentage and calculated as follows:

$$NR = ((C - T)/N) * 100$$

Where:

C = worms observed in the control soil;

T = worms observed in test soil;

N = total number of worms per replicate;

A positive (+) net response indicates avoidance and a negative net response (-) indicates a non-response (or attraction) to the chemical or different soil tested. In accordance with the previously mentioned Draft Guideline for the Earthworm Avoidance Text (ISO 2004), the habitat function of soils is considered to be limited if on average > 80 % of worms are found in the control soil (indication of an impact on behaviour).

3. Results

Avoidance to different soil types (Fig. 1)

The results indicate a clear preference of the worms for LUFA 2.2 compared to six out of 10 soils (including OECD soil). Only in the Sch3 and in the Coi3 soils nearly the same number of worms was found in either these soils or in LUFA 2.2, while ESo2, Tau4 and ESo5 seem to be acceptable but not preferred. In the tests with OECD artificial soil as control, three soils (LUFA 2.2, Coi3 and Tau4) were clearly preferred. ESo2, Sch3 and Mon4 had an intermediate status. When compiling the results of all tests together, some 80% of all worms preferred LUFA 2.2 and about 60% of all worms preferred OECD artificial soil compared to the respective field soils.

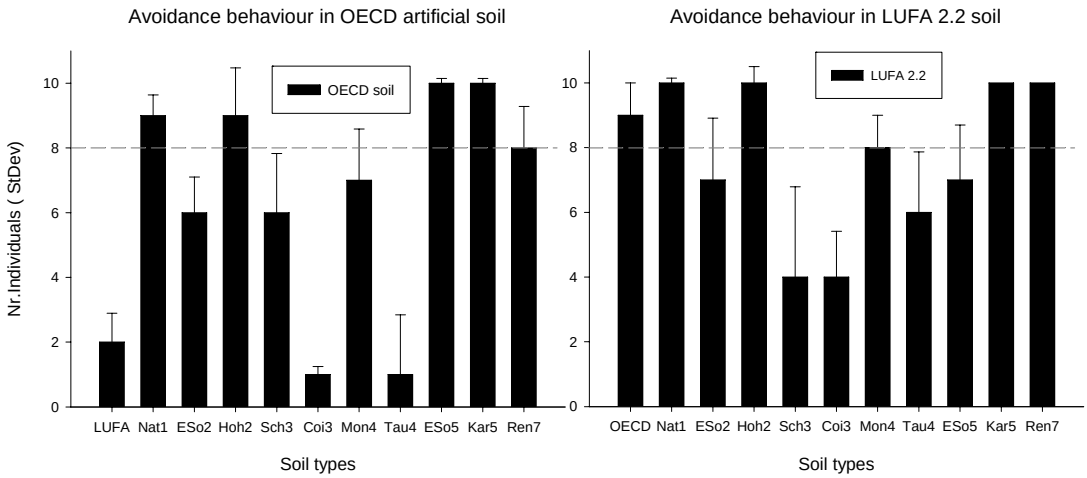


Figure 1: Results of the avoidance test performed with Enchytraeus albidus, when exposed to OECD artificial soil and LUFA 2.2 soil (as reference soils) versus different soils. Results express average values ± Standard Error.

<u>Avoidance to Benomyl</u> (Fig. 2)

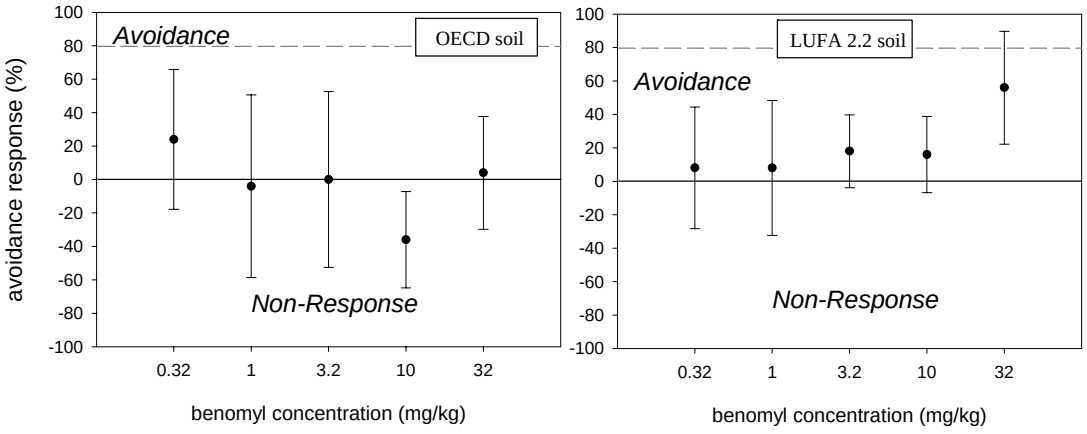


Figure 2: Results of the avoidance test performed with *Enchytraeus albidus*, when exposed to OECD artificial soil and LUFA 2.2 soil, in a control versus contaminated situation with the test item Benomyl. Results express average values ± Standard Error.

The results indicate an avoidance behaviour at high concentrations in LUFA 2.2 soil (EC50 = 46.8 mg/kg) whereas in OECD soil there is no evidence of an effect in the tested concentrations.

3) Avoidance to Carbendazim (Fig. 3)

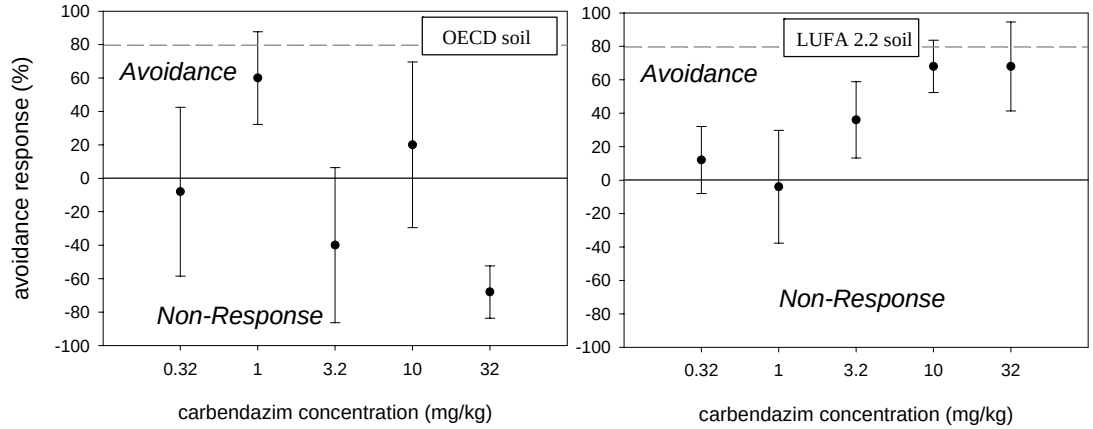


Figure 3: Results of the avoidance test performed with *Enchytraeus albidus*, when exposed to OECD artificial soil and LUFA 2.2 soil, in a control versus contaminated situation with the test item Carbendazim. Results express average values ± Standard Error.

The results show avoidance behaviour already at medium concentrations in LUFA 2.2 soil (EC50 = 7.9 mg/kg), while it is not possible to detect a clear tendency in OECD artificial soil.

4) Avoidance to Phenmedipham (Fig. 4)

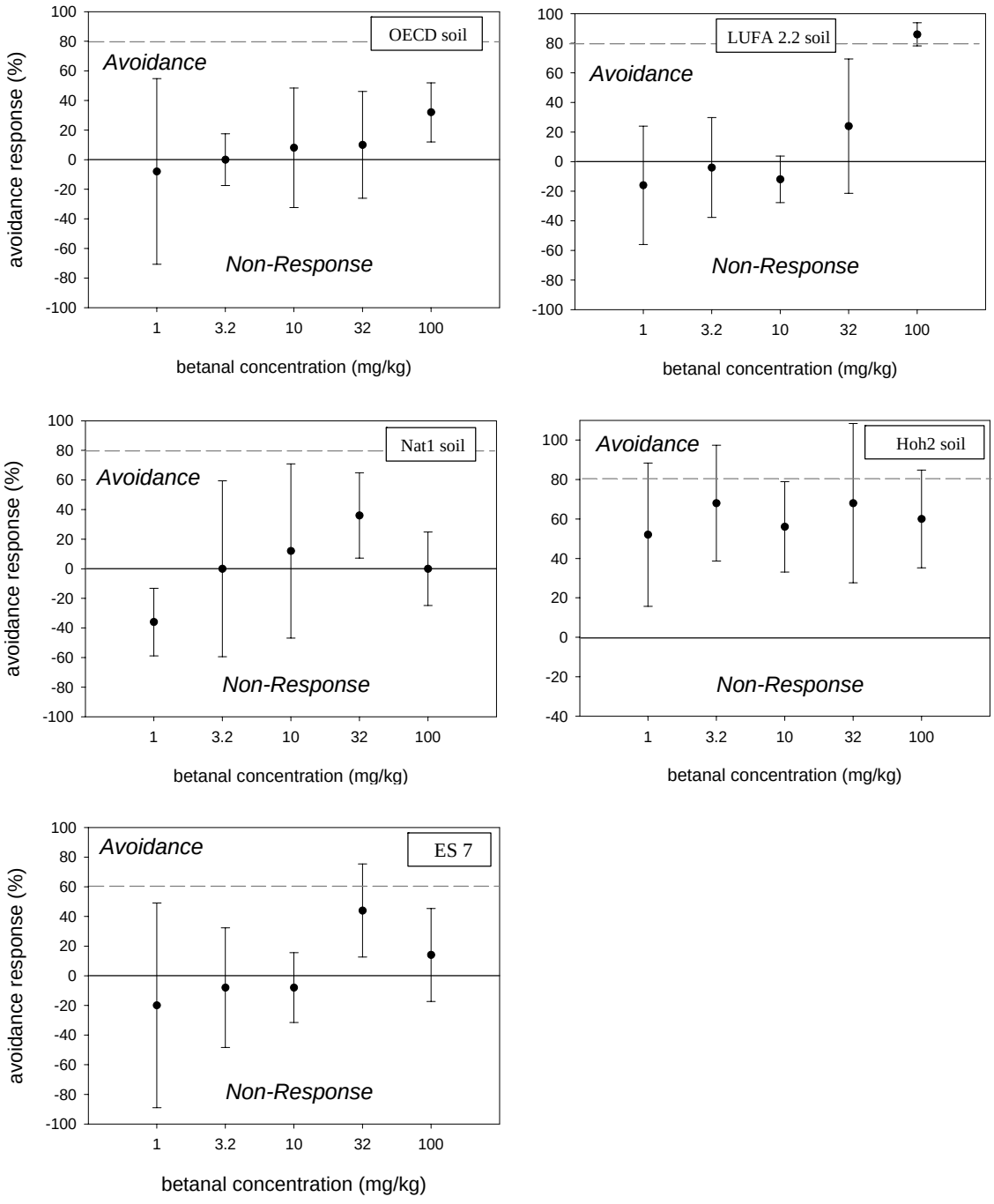


Figure 4: Results of the avoidance test performed with *Enchytraeus albidus*, when exposed to OECD artificial soil, LUFA 2.2 standard soil, Nat1, Hoh2 and ES7 soil, in a control versus contaminated situation with the test item Phenmedipham. Results express average values ± Standard Error.

The results show higher effects in LUFA 2.2 soil (EC50 = 50.7 mg/kg) then in OECD soil (estimated EC50 = 252.2 mg/kg). However, in the latter case this value is extrapolated clearly above the tested concentration range.

Avoidance to Phenmedipham in Nat1 soil (Fig. 4)

Avoidance behaviour can be observed at a concentration of 32 mg a.i./kg in soil Nat1 (EC50= 45.9 mg/kg). The highest concentration apparently induced narcosis, reflexes were disturbed and the worms were unable to escape to the control side. Therefore, this concentration was excluded from the statistical evaluation.

Avoidance to Phenmedipham in Hoh2 soil (Fig. 4)

Avoidance behaviour started already at the lowest tested concentration (1 mg a.i./kg) and this situation did not change at all higher concentrations. Therefore, no EC50 calculation was possible. However, it can be assumed that 50% of the test organisms were already affected at about 1 mg a.i./kg.

Avoidance to Phenmedipham in Euro Soil 7 (Fig. 4)

As in the case of the soil Nat1, avoidance behaviour was observed at the second-highest concentration (32 mg a.i./kg) , while at the highest concentration narcosis was induced, which leads to a non-dose-response type of reaction. By excluding the effect value at 100 mg a.i./kg for this reason, the EC50 was calculated as 62.7 mg a.i./kg.

Table 3: EC_{50} (mg/kg) values of the experiments performed with the toxic substances.

Experiment	Avoidance EC50 (mg/kg)	Survival LC50 (mg/kg)	Reproduction EC50 (mg/kg)
LUFA 2.2 / Benomyl	46.8	1.8	1
OECD / Benomyl	> 32.0	25.7	5
LUFA 2.2 / Carbendazim	7.9	2.5 #	0.8 #
OECD / Carbendazim	> 32.0	4.9	3.7
LUFA 2.2 / Phenmedipham	50.7	56.6	31
OECD / Phenmedipham	252.2	> 100	46
Nat1 / Phenmedipham	45.9a)	> 32 < 100 *	++
Hoh2 / Phenmedipham	< 1.0	> 100 *	++
ES7 / Phenmedipham	34.2a)	> 32 < 100 *	++

* = estimated based on the results of the avoidance tests; ++ = reproduction not possible due to the natural properties of these soils); # = estimated based on a test with a loamy field soil (16) a) highest concentration eliminated for calculations.

The results of the avoidance tests performed here are compared to our own results from acute and chronic tests as well to literature data (Römbke 1989; Römbke and Federschmidt 1995) (Tab. 3). According to this compilation, one can conclude the following:

- Enchytraeids reacted clearly more sensitively to the test chemicals when tested in LUFA than in OECD soil;

- When using field soils, a broad range of sensitivity was determined;

- The EC50 values for avoidance were higher than the EC50 values for reproduction;

- In comparison to the acute LC50 values, the results of the avoidance tests are mostly in the same order of magnitude.

Regarding the reactions in different soils to the same chemical, no clear correlation between the sensitivity towards Phenmedipham, and certain soil properties could be identified.

4. Discussion

Actually, enchytraeids have so far been very rarely used in avoidance tests. In fact, the first attempts to their use came up in the mid-Nineties, focusing on the species *Enchytraeus crypticus* and using avoidance as well as escape behaviour as endpoints (Achazi *et al.* 1996). This test was mainly performed for the evaluation of contaminated soils (Achazi *et al.* 1999), but recently single chemicals were also investigated in LUFA 2.2 soil (Römbke *et al.* 2000), where some heavy metals (cadmium, copper) and pesticides were studied (Ethiofencarb, Carbofuran, Cypermethrin and Phenmedipham).

Methods

In previous studies performed with earthworms, different types of equipment and techniques were used, e.g. circular test chambers divided in two sides and various test durations (between one and seven days) (Yeardley *et al.*, 1996), or circular test chambers divided into 6 pie-shaped sections, where different toxic concentrations in soil are tested simultaneously, surrounding a central circular chamber where the worms are introduced (observations at 24 and 72 hours) (Stephenson *et al.*, 1997). Both approaches are part of the earthworm ISO Draft (ISO 2004). The experience gained in this study does not support the idea to change the two-chamber test vessels in an enchytraeid avoidance test. However, the duration of 48 h might be questionable since in the light of the relative low sensitivity modifications might be helpful (e.g. an even shorter test period).

Avoidance of different soils

When assessing the different soil preferences it was observed that most of the times LUFA 2.2 soil was preferred as a control compared to OECD soil; probably because this natural soil fulfils the needs of *Enchytraeus albidus* better than the OECD artificial soil. In addition, higher average numbers of juveniles were obtained in reproduction tests with LUFA 2.2 soil in comparison to OECD soil. Therefore, OECD artificial soil seems to be less appropriate for the performance of enchytraeids tests, hence LUFA 2.2 is proposed as the control soil of choice in this avoidance test.

Referring to the 80% criterion, the habitat function of three out of 10 field soils is not limited for soils Coi3, Sch3 and Tau4, while the other soils (Nat1, Hoh2, Mon4, ESo5, Kar5, Ren7 and ESo2) were clearly avoided by the enchytraeids. In the case of the soils belonging to the EURO-Soil Class 5 and 7, these results are probably caused by their low pH values. This hypothesis was confirmed in previous studies (Amorim et al, 1999), where reproduction of *E. albidus* did not occur at pH values lower than 4.6. In addition, in an avoidance study using *Eisenia fetida* with seven different soils (Hund-Rinke and Wiechering, 2001), the worms showed a tendency to escape only from an acid forest soil (pH of 4.1). The reasons for rejection and preference for other soils are not so obvious. While the high clay content in Nat1 and ESo2 might have played a role, no reason could be found for avoiding the soil Mon4. In reproduction studies (Amorim *et al.* in preparation) performed with *E. albidus* in different soils, the results indicated that the worms survive but did not reproduce in soils belonging to Euro-Soil Nos. 1, 2, 5 and 7, probably due to their high clay content (Nos. 1 and 2) or low pH (Nos. 5 and 7).

Since the results of the reproduction and avoidance tests are in good accordance, avoidance tests can be used to determine whether a field soil to be assessed does have toxic properties even without any anthropogenic contamination (e.g. in terms of pH). In this respect the short-term avoidance test could act as a screening test before starting a long-term reproduction test. However, due to some unexpected results, further studies are needed to understand the influence of soil properties on the avoidance behaviour of enchytraeids.

Avoidance to different chemical substances

Generally speaking, avoidance behaviour could be observed with increasing toxic concentrations. Also, a clearer and more sensitive response could be gained from the studies with all three model chemicals in which LUFA 2.2 natural soil had been used as a control medium. The same observation was made by Garcia (2004) who determined EC50 values for *Eisenia fetida* in LUFA and OECD soils for Benomyl (1.6 versus 28.2 mg a.i./kg) and Carbendazim (7.1 versus 127.4 mg a.i./kg). As mentioned earlier, OECD soil is less suitable for the worms. In addition, its high organic matter content might be responsible for a lower bioavailability of adsorbing the test chemicals. As a result, the chemical becomes less noticed by the worms. Therefore, the chemical is less avoided or, more unequally distributed than is in the LUFA soil. In the latter case, the worms may be trapped in a “hotspot” or else may not realise that there is a contaminant at all.

The observations related to the use of OECD artificial soil are in accordance with the results of several other enchytraeid and earthworm studies (e.g. Belfroid and Sijm, 1998, Martikainen, 1996, Amorim *et al.*, 2002a) b). They show that the high organic matter content and therefore the high adsorptive capacity to hydrophobic chemicals of OECD artificial soil leads to an underestimation of the toxicity of such chemicals in comparison to many natural soils. For this reason, a discussion is currently going on whether the organic matter content of OECD artificial soil should be lowered to 5 % instead of 10 %. However, any change in using a standard test substrate must be very well substantiated due to the high amount of comparable information which has been gained with the OECD soil within the last 20 years.

For Benomyl and Carbendazim, the avoidance EC50 values were in the same order of magnitude as the mortality LC50 values, but clearly higher than the respective EC50 values for reproduction. This contrasts with the conclusions of Garcia (2004), where this situation is different in the case of earthworms where the EC50 values for avoidance are clearly lower than mortality but in the same order of magnitude for

reproduction. A similar observation has been made when testing differently contaminated field soils with earthworms (Hund-Rinke *et al.*, 2003). The reasons for this different behaviour (i.e. less sensitivity) in comparison to earthworms are not yet clear. As far as one can say on the basis of a limited data set (e.g. for earthworms: Edwards and Bohlen (1992); for enchytraeids: Didden and Römbke 2001), there is no consistent difference in sensitivity between these two organism groups. Thus, the specific properties of the two fungicides may play a role, but only more data with different chemicals will help to answer this question. However, it is important to remember that this kind of test may not be useful for all compounds, especially for those that show no irritant effect or those which do have a narcotic mode-of-action, as described by Yeardley *et al.* (1996). Notwithstanding, this does not influence the conclusion that the enchytraeids avoidance test can act as a screening test to investigate the need for further more time-consuming and costly tests.

For Phenmedipham tested in OECD and LUFA soils, the effect levels are similar with regard to mortality and avoidance, but both are clearly higher than in the reproduction test. Again, the avoidance test may be used as a screening test for acute and chronic tests, in particular when a reproduction effect is not possible due to natural soil properties. This could be confirmed in this study, where avoidance behaviour but not reproduction was determined in the natural soils Nat1, Hoh2 and ES7. Different effect levels depending on the soil were observed: Phenmedipham was much more toxic in Hoh2 than in ES7 and Nat1. No correlation with a certain soil property could be identified in order to explain this result.

Table 4: Comparison of test results with Phenmedipham in Lufa 2.2 soil and two enchytraeid species.

Species	Avoidance	Survival	Reproduction

	EC_{50} (mg/kg)	LC_{50} (mg/kg)	EC_{50} (mg/kg)
Enchytraeus albidus	50.7	56.6	31.0
Enchytraeus crypticus	20.0	74.8	36.9

In the case of the herbicide Phenmedipham, a comparison of the results with *E. crypticus* and *E. albidus* is possible (Table 4), despite the fact that the test designs were not completely similar (e.g. the acute test with *E. crypticus* was only running for 7 days). According to this first comparison the results are in the same order of magnitude. The more sensitive reaction of *E. crypticus* in the avoidance test is caused by the fact that this test was done as an escape test, i.e. the animals were put on the spiked soil and not on the borderline between treated and control soil, as in the case of the test with E. albidus. So, one can conclude that in the case of Phenmedipham the different endpoints are relatively closely together, with mortality being the most insensitive endpoint.

As concluding remarks, *Enchytraeus albidus* (and, referring to literature data, *E. crypticus* as well) seems to be a suitable test organism for avoidance testing. LUFA 2.2 soil appears to be the best choice as a control for substrate or chemical testing instead of OECD artificial soil, and so it may be used as a control in further similar bioassays.

Avoidance tests are useful as screening tools for the assessment of potentially contaminated soils or of chemicals in soils. In addition, they are valuable to evaluate the influence of soil properties on these worms. Provided that a larger data base concerning the relation between avoidance and mortality/reproduction data is available, an avoidance test could be a time-saving alternative to long term tests.

Due to these reasons and not withstanding the need for further testing using other chemicals and soils, this test should be standardized and recommended for effect evaluation along the lines already discussed for the Earthworm Avoidance Test (ISO 2004).

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Chapter 5

Effect of different soil types on the enchytraeids *Enchytraeus albidus* and *Enchytraeus luxuriosus* using the herbicide Phenmedipham

5. Effect of different soil types on the enchytraeids *Enchytraeus albidus* and *Enchytraeus luxuriosus* using the herbicide Phenmedipham

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ABSTRACT

Soil ecotoxicology studies are usually performed in standard soils, such as the OECD artificial soil or the LUFA St. 2.2, a natural soil. When assessing the toxic effects in the environment, soil properties are often different from those in standard soils, which might lead to a different exposure situation for the test species and, therefore, to a different evaluation of the risk of the test substance. Selected to cover a broad range of properties and based on the Euro Soils concept, 16 different soils were studied regarding their suitability to two test species: *Enchytraeus albidus* and *Enchytraeus luxuriosus* (Enchytraeidae). In reproduction tests, the test species reacted differently to the tested soils, but both enchytraeids did not survive in acid soils (i.e. pH ≤5). In the main test, the effects of the reference substance Phenmedipham (formulation Betosyp) on the enchytraeids were studied in those soils in which sufficient reproduction was determined beforehand. Results indicate that although an additive and/or synergistic effect of soil properties seems to occur, certain soil properties are causing specific toxic effects. In the present study, EC_{50}s in enchytraeids changed by a factor of 9 for juveniles and nearly 30 for the adults of *E. luxuriosus* (maximum values; slightly lower values were found for *E. albidus*), which shows how important the test soil can become for the environmental risk assessment of chemicals. More data using more soils and species are required to understand the effect of soil properties in soil toxicology. Nevertheless, it was clear that certain soil properties such as OM and WHC or pH, CEC, C/N and clay content did interact with the chemical and the organisms.

Keywords: Natural soils, laboratory tests, EURO-Soils, reproduction.

1. Introduction

Environmental effects of chemicals can only be understood if their fate, metabolism and interactions with the components of the environmental compartment to be protected are known. The best way to achieve a comprehensive insight into these complex processes is certainly through long-term studies in the respective ecosystems (Gawlik *et al.*, 1996). However, due to practical restrictions (e.g. limited resources) the risk of chemicals is assessed by using a tiered system, starting with simple laboratory tests. In soil ecotoxicology, it is common to use standard soils in laboratory tests such as the artificial OECD soil (OECD, 1984) or the natural standard LUFA 2.2. The results of such tests are difficult to extrapolate to the real field situation, given the fact that natural soils have different properties that can influence the fate and the effects of the test chemical. Therefore, the use of standard soils might lead to an inaccurate environmental risk assessment. Van Gestel and Ma (1988, 1990) determined LC_{50} values in four different soils, using *E. andrei* and *Lumbricus rubellus* as test species. These values differed by a factor of 4.4 to 12.8, being reduced to a factor of 1.2 to 2.4 when recalculated to pore water concentrations. Despite the fact that the authors concluded that these LC_{50}s were mainly determined by the concentrations in the soil pore water, which can be easily predicted from the sorption data, further studies from Belfroid (1994) concluded that this was not linear and that the pore-water hypothesis is useful only as a starting point. Also, Løkke (1994) stated that it is not justified that only the water soluble fraction of the chemical in question is bioavailable. Therefore, the study of the different properties of soils and its interaction with chemical substances and different species is an important issue which remains still inconsistently answered.

A set of reference soils – the so-called EURO-Soils – was introduced in 1990 to create a common basis for a better comparison and quality control of soil sorption data (Kuhnt et al., 1994a). Firstly five, finally six regionally representative soils were identified, collected, prepared and characterised as reference soils for chemical testing in the EU (Gawlik et al., 1996). The demand for EURO-Soils grew dramatically after their introduction, and it became quickly evident that the original

EURO-Soils are not a suitable source for ecotoxicological standard tests simply due to the fact that the available amount is just not sufficient for the demand.

Recently, Römbke and Amorim (2004) suggested that each soil similar in terms of their main properties (i.e. texture, pH, C/N ratio and organic matter content) to one of the six EURO-Soils could be used for ecotoxicological tests. To validate this hypothesis, such tests were performed for comparison purposes in the original Euro Soils, similar soils (SIM Soils) and the standard OECD and LUFA 2.2 soils. While several species were chosen as test organisms, here we reprot the results with the two enchytraeids *Enchytraeus albidus* and *Enchytraeus luxuriosus*.

Enchytraeids (Oligochaeta) are important members of the soil biocenosis and are typical inhabitants of many soils, contributing to vital processes of this environmental compartment. Indirectly they are involved in regulating the degradation of organic matter, as well as improving the pore structure of the soil (Didden 1993). Additionally, there is a standardised toxicity test available for the selected test species (ISO 2003). To determine the ecological requirements of *Enchytraeus albidus* and *Enchytraeus luxuriosus*, these species were tested in the original EURO-Soils and some soils similar to them (the so called SIM-Soils). In addition, the commonly used OECD artificial and LUFA 2.2 standard soils were used. Reproduction, which is undoubtedly ecologically of utmost importance (Straalen *et al.* 1994), was used as the main endpoint in these chronic tests with untreated soils. Afterwards, the same reproduction tests were performed to evaluate the potential influence of the different soils on the test effects of the test substance Phenmedipham. This substance was selected due to its known properties and toxicity to soil invertebrates and because it has been used for several years as a reference substance in a ringtest organised by the German Federal Environmental Foundation (Hund-Rinke et al. 2002).

2. Materials and Methods

Test organisms

Two test species *Enchytraeus albidus* Henle, 1837 and *Enchytraeus luxuriosus* Schmelz & Collado, 1999 were used. *E. albidus* is one of the largest species of the genus *Enchytraeus* (adults reach 15 – 40 mm) while *E. luxuriosus* (adults reach 8 – 13 mm) is much smaller. Both species were maintained in laboratory cultures, being bred in moist soil (50% OECD soil, 50% natural garden soil), at 20ºC, kept in the dark and fed once a week with finely ground and autoclaved rolled oats (Cimarrom, Portugal). Details of the culturing process are given in Römbke and Moser (2002).

Test Substance

The test substance Phenmedipham, an herbicide, was applied as the formulation Betosyp (formerly known as Betanal; Stähler Agrochemie, 157g/l a.i.) to the soils in the following concentrations: 1, 3.2, 10, 32 and 100 mg a.i./kg soil DW. The contamination of all test substrates was done by mixing an aqueous solution of the test chemical into the pre-moistened soils, separately for each test concentration. After homogeneous mixing, sub-samples of soil were introduced into the individual test vessels.

Test soils

The main properties of the test soils (pH, Organic Matter, Carbon/Nitrogen, Cation Exchange Capacity, Water Holding Capacity, clay, silt and sand content) are given in Table 1. Their selection is described in detail by Römbke and Amorim (2004).

Artificial soil (OECD, 1984) is constituted by 69% sand, 20% kaolin clay, 10% sphagnum peat and 0.3 to 1% of $CaCO_3$ for pH adjustment (6±0.5). LUFA 2.2 is a natural standard soil from Speyer (Germany). The codes given for the natural soils are translated as follows: ES means EURO-Soil, ESo means that the soil is a sample from the same site as the original ES, the numbers mean that the soil is similar to a certain ES number (X = the soil could not classified to a certain ES class) and the other codes represent the first three letters of the soil original place name: Nat1: Natzungen; Hoh2: Hohenlimburg; Coi3: Coimbra; Sch3: Schmallenberg; Mon4:

Mönninghausen; Tau4: Taubenheide; KarX: Karlsruhe (Schluttenbach); Ren7: Gladbeck-Rentfort. At least one natural soil from each class was tested. Not surprisingly, it was most easy to find soils belonging to ES class 3, since this class represents "normal" agricultural soils in Central Europe. In some cases, in particular the original EURO-Soils, the amount of available soil was not enough to perform all tests. With the exception of soil ES7, bought from the University of Vienna (Austria), the other samples came from the European Chemical Bureau (Ispra, Italy), where only a small rest amount remained available.

Table 1: Main characteristics of the tested soils and the relative EuroSoils (ES) properties: pH, OM, C/N, grain size distribution, CEC and WHC.

SOIL	pH ($CaCl_2$)	O.M. (%)	C/N	Clay (%)	Silt (%)	Sand (%)	CEC (mval/100g)	WHC (%)
ES1	5.1	2.7	7.7	75	22	3	29.9	62.6
Nat1	6.2	1.7	8.7	33	66	5	40.7	58.4
ES2	7.4	6.4	18.5	23	64	13	28.3	68,5
Hoh2	6.2	12.9	25.0	6	61	33	78.3	73.9
ES3	5.2	6.5	13.3	17	37	46	18.3	42.6
Sch3	5.4	4.1	10.4	23	45	32	68.5	67.4
Coi3	6.7	6.5	17.0	26	60	14	75.8	68.1
LUFA 2.2	5.8	4.4	14.0	6	17	77	11.2	55.0
ES4	6.5	2.9	9.7	20	76	4	17.5	42.9
Mon4	6.5	2.5	9.7	11	77	12	20.7	53.2
Tau4	6.9	2.9	9.7	17	79	4	61.3	63.1
ES5	3.2	15.9	30.8	6	13	81	32.7	38.7
Eso5	3.2	9.2	29.7	10	12	79	87.0	100.1
ES7	4.4	11.5	14.2	19	35	46	5.0	80.6
Ren7	3.8	8.7	11.0	18	40	42	132	121.8
ESoX	6.3	8.9	23.5	31	33	36	-	64.0
KarX	3.6	10.6	45.9	13	58	29	173	71.9
OECD artificial	6.0	8.0	Ca. 40	10	10	80	45.8	Ca. 90

Experimental procedure

The Enchytraeid Reproduction Test (ERT) was standardised in 2003 for the study of single chemicals as well as contaminated soils (ISO 2003; OECD 2003). Ten adult worms with well developed clitellum were introduced in a glass vessel, each containing 25 g moist soil plus food supply (finely ground and autoclaved rolled oats; 0.5 mg for *E. albidus* and 0.25 mg for *E. luxuriosus,* being half of the amount

supplied every week). Four replicates per treatment were used. The duration of the tests with *E. albidus* was six weeks: after three weeks the adults were gently removed and the soil was left for three additional weeks for juveniles to hatch and grow. The test duration for *E. luxuriosus* was 4 weeks and the adults were left in the vessels until the end of the test. At the end of the test, the organisms' were immobilisized with alcohol and coloured with Bengal red. After some hours the organisms are coloured and the soil solution was spread in a box and observed under the binocular for counting. Mortality of adults and the number of juveniles were evaluated for both species.

Experimental setup

The whole study was done in two parts: In the first set of experiments the survival and reproduction of the two species was tested in all soils in a control situation, i.e. without any contamination. The aim was to verify the suitability of the individual soils for a certain species. In the second set of experiments, only the soils in which reproduction was within the validity range (mortality < 20% and number of juveniles > 25 per test vessel) as defined in the ISO guideline (2003) were tested.

Statistics

There are two main hypothesis to be tested. It is hypothesised that the measured soil properties influence:

1) The survival and reproduction of the test organisms (tests without chemicals);
2) The toxicity of the toxic compound, either by directly altering the exposure (e.g. due to different adsorption and bioavailability) or by adding an extra stress factor for the organisms (in addition to the chemical).

Redundancy Analysis (RDA) was applied to the results of survival and reproduction of enchytraeids and collembolans maintained in different soil types in the absence of toxicants. The analysis was performed with Canoco for Windows 4.5 (Ter Braak and Smilauer, 2002) using survival and reproduction of each species to play the role of species, and the physical and chemical parameters of each soil to play the role of environmental data. All data was log-transformed prior to the analysis, except for pH values. Additionally only the two extreme categories (sand and clay, excluding silt)

of the three texture classes due to the interdependence of the individual parameters were used. Species data was centred and normalised within Canoco for Windows. A similar procedure was used to analyse toxicity data from exposure of enchytraeids in different soil types. However, in this case toxicity parameters (EC_{50} and NOEC) were used to play the role of species. Due to experimental design constrains (e.g. limited amount of soil available or unsuitability of a soil type for a given species) it was not possible to expose all species to the same soil types, thus a separated analysis was conducted for each species. Conditional effects of environmental data on species data were assessed using Monte Carlo permutation tests with automatic variable selection from within Canoco for Windows.

Stepwise Multiple Regression models were developed, using the statistical software package SPSS 12.0 (SPSS, 2003), to quantify the relationship of the biological data with soil data. All but the pH was also normalised using logarithms (X+1) and silt was excluded. Furthermore, ANCOVA analysis (Zar, 1996) showed that regression lines between species are coincidental for adults and juveniles in enchytraeids as control.
Analysis of variance was calculated using SPSS 12.0. EC_{50s} and NOECs were calculated using the ToxRatPro program (ToxRat 2003).

3. Results

First set: Control experiments

In total, the mortality and reproduction of *E. albidus* was tested in 16 soils plus two control substrates (Fig. 1). Major differences in the mortality and reproduction were found in these tests: in some soils where survival and reproduction were impeded completely (e.g. ES5, ESo5) and in a few others survival of the worms was possible but not reproduction (Nat1, ESoX, Hoh2, ES4 and ES7). The validity criterion for mortality (< 20%) was met in +/- 12 soils, but for the endpoint reproduction this number was lower: only in nine soils more than 25 juveniles per test vessels were observed. The highest number of juveniles was found in the standard OECD and

LUFA 2.2 soils (60 and 90, respectively). With the exception of ES2, Coi3, Sch3 and Tau4 statistical significant differences (One way ANOVA, Dunnetts' two sided; $p<0.05$) occurred between OECD and most of the soils. In nearly all cases a high variability of mortality and juvenile numbers was measured.

The results of the tests with the second species, *E. luxuriosus*, were similar to the ones with *E. albidus* (Fig. 2). Even more soils were completely (or nearly) impeding survival or reproduction: ES5, ESo5, ES7 and KarX. In another seven soils the worms survived but could not reproduce (Nat1, ESoX, Hoh2, ES4, Mon4 and Tau4). The validity criterion for mortality (< 20%) was met in +/- 11 soils, but for the endpoint reproduction this number was lower: only in six soils more than 25 juveniles per test vessels were observed. The highest number of juveniles was found in the natural soil Sch3 (60), while in the standard OECD and LUFA 2.2 soils just about 25 juveniles were observed. Statistical significant differences (One way ANOVA, Dunnetts' two sided; $p<0.05$) occurred between OECD soil and the natural soils ES1, Nat1, ESoX, Hoh2, Mon4, Tau4 and Ren7 (plus those soils in which no juveniles were found at all).

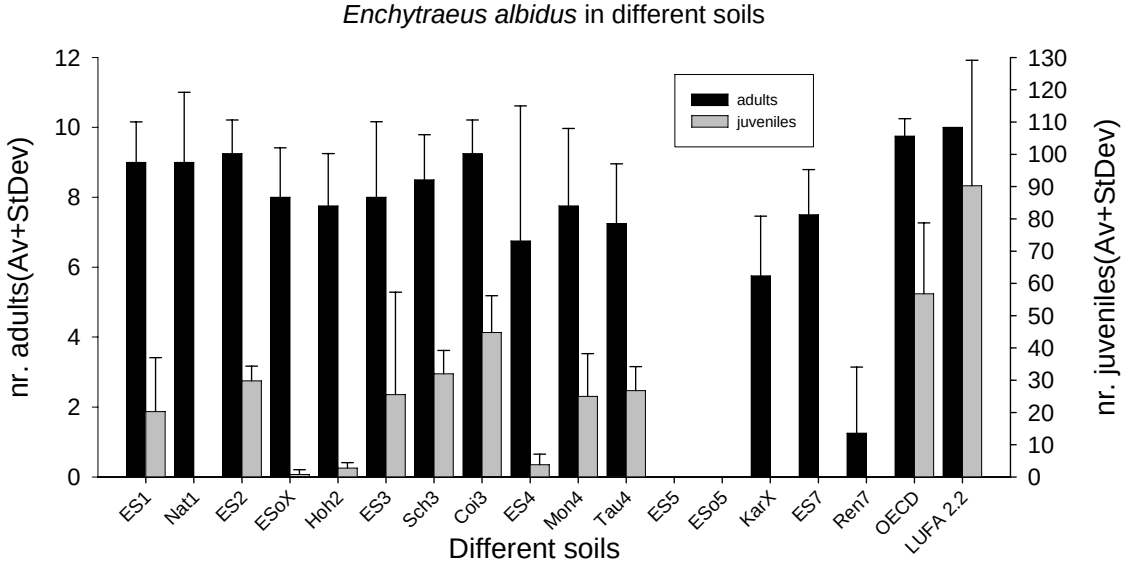

Figure 1: Results obtained exposing *Enchytraeus albidus* to different soil types (OCDE soil, LUFA2.2, Euro-Soils and similar soils). Graphs show the average number of organisms + standard deviation.

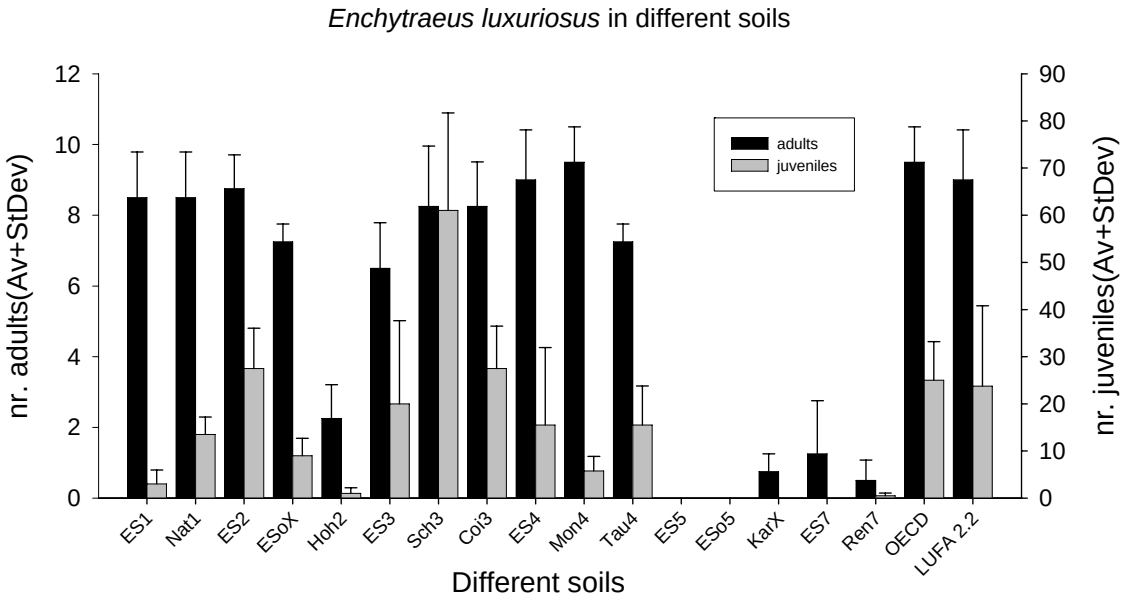

Figure 2: Results obtained in exposing *Enchytraeus luxuriosus* to different soil types (OECD soil, LUFA2.2, Euro-Soils and similar soils). Graphs show the average number of organisms + standard deviation.

Test substance experiments

In the case of *E. albidus* and of *E. luxuriosus* six and seven soils (including the two standard soils) were tested with the test item Phenmedipham (as Betosyp). This number is lower than the soils in which sufficient reproduction was observed in the first set, since the amount of samples was not sufficient to run all tests. The results of these experiments can be seen in Figures 3 and 4 as well as Table 2.

The effects on the adult worms varied between 48.5 and 125.6 mg a.i./kg (EC_{50}) and 32 and > 100 mg a.i./kg (NOEC) in the test with *E. albidus*, i.e. the factor between minimum and maximum is relatively small (a factor of three; i.e. in the case of NOECs just one concentration apart). In the tests with *E. luxuriosus* this factor is higher (5 – 30). Consequently, the individual values differed more: 28.6 and 147.4 mg a.i./kg (EC_{50}) and 1.0 and > 32 mg a.i./kg (NOEC). In addition, these results prove a higher sensitivity of the adults of the latter species towards Phenmedipham. Based on EC_{50} values, for both species the lowest toxicity was found in OECD artificial soil, followed by ES2 soil (and ES1 in the test with *E. albidus*). Taking the

NOECs into consideration, the toxicity in LUFA St. 2.2 soil was also relatively low.

Table 2: Resulting EC_{50}s and NOECs from the exposure of the 2 different species to Phenmedipham. (n.d.=not determined due to mathematical reasons; n.t.=not tested;).

Soils	*Enchytraeus albidus*				*Enchytraeus luxuriosus*			
	EC_{50}		NOEC		EC_{50}		NOEC	
	Ad	**Juv**	**Ad**	**Juv**	**Ad**	**Juv**	**Ad**	**Juv**
OECD	>100	34.5	≥100	32	147.4	42.3	32	1.0
LUFA 2.2	49.8	29.4	32	10	29.4	31.7	32	10
ES1	>100	13.9	≥100	10	n.t.	n.t.	n.t.	n.t.
ES2	125.6	17.5	32	3.2	93.4	33.2	3.2	10
ES3	105.2	56.6	32	32	n.d.	n.d.	3.2	32
Sch3	n.t.	n.t.	n.t.	n.t.	5.6	6.1	1.0	1.0
Coi2	48.5	22	32	10	43.7	n.d.	32	<1.0
ES4	n.t.	n.t.	n.t.	n.t.	28.6	49.1	3.2	32
Tau4	n.t.	n.t.	n.t.	n.t.	32.5	5.5	32	<1.0

The EC_{50}s for juveniles are ranging from 13.9 to 56.6 mg a.i./kg in *E. albidus* and from 5.5 to 49.1 mg a.i./kg in *E. luxuriosus*, indicating a factor of four for the former and a factor of nine for the latter species between minima and maxima. The respective NOEC values showed the same relationship: In *E. albidus* all results varied between 3.2 and 32 mg a.i./kg, while for *E. luxuriosus* the factor was again higher, since the NOEC values differed between 1.0 and 32 mg a.i./kg. Using this endpoint, OECD artificial soil was not the substrate showing the absolute lowest toxicity – natural soils like ES3 or ES4 revealed slightly higher EC_{50} values.

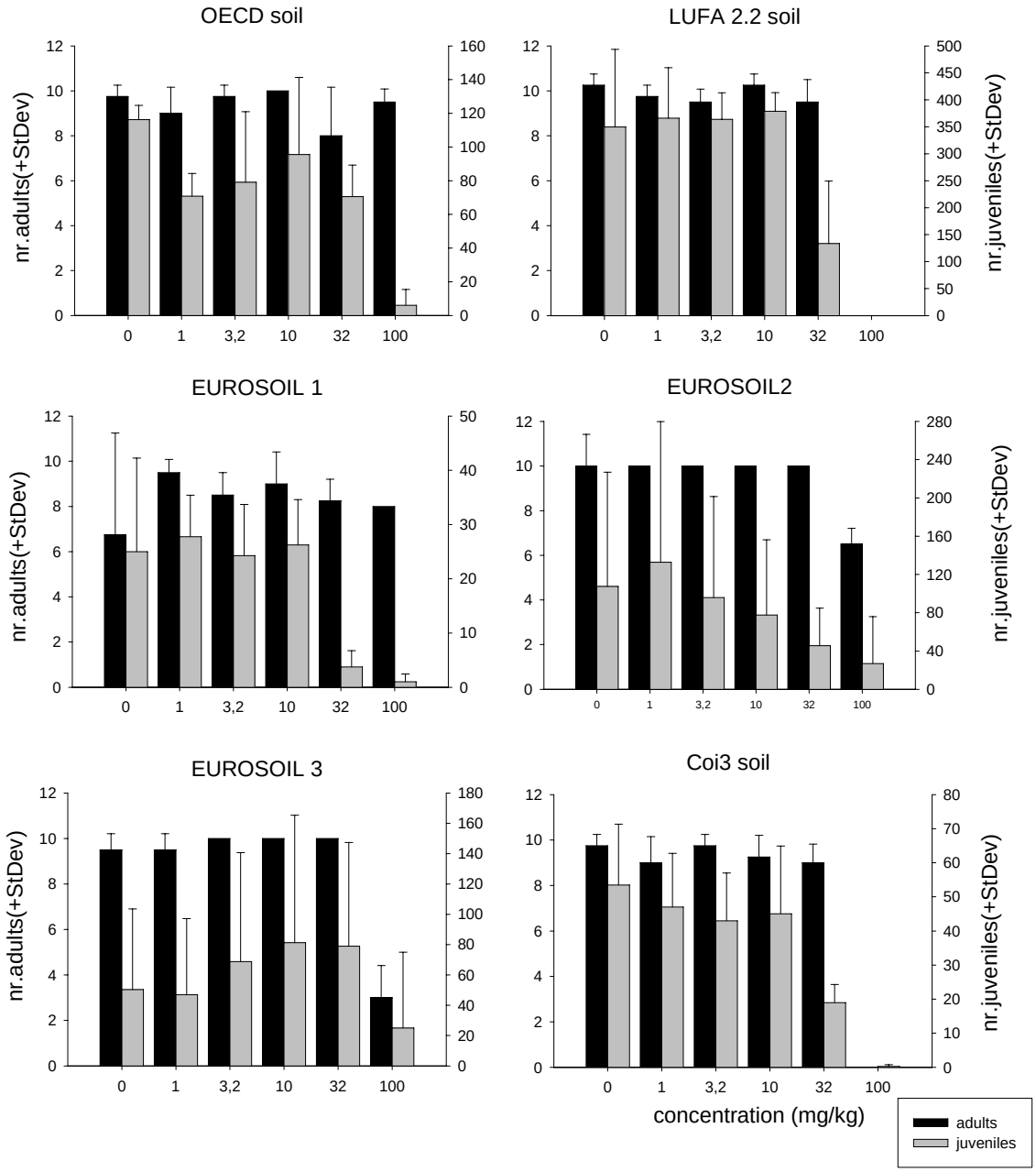

Figure 3: Results obtained by exposing *E. albidus* in different soils to Phenmedipham. Graphs show the average number of organisms + standard deviation.

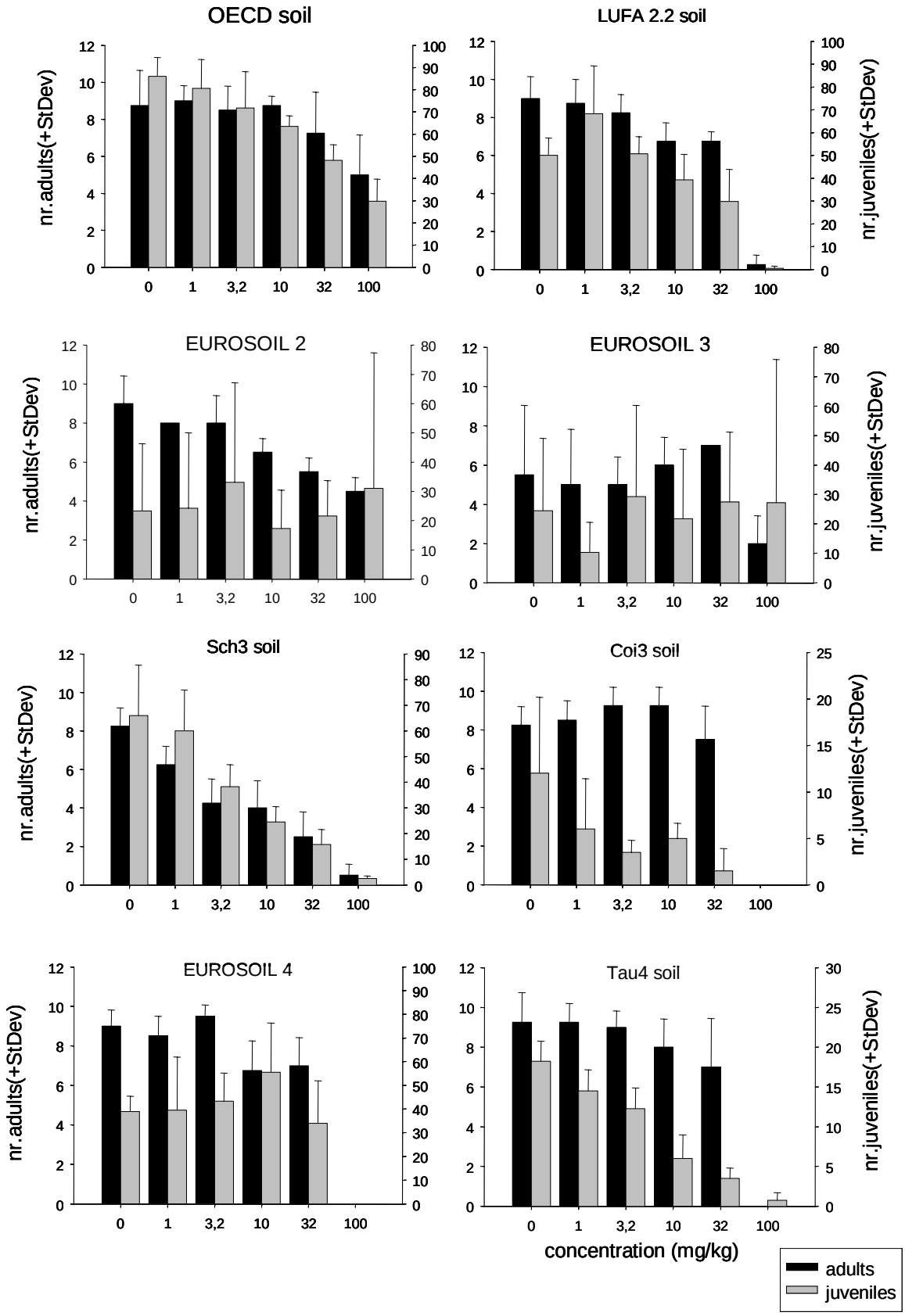

Figure 4: Results obtained by exposing *E. luxuriosus* in different soils to Phenmedipham. Graphs show the average number of organisms + standard deviation.

Interestingly, in the tests with *E. albidus* the NOEC for reproduction was always lower than the NOEC for survival, but in the tests with *E. luxuriosus* this (expected) relation differed: in three out of five tests adult survival was the more sensitive endpoint. One might assume that these three cases were artefacts due to a wrong selection of the test concentrations, but in one of them also the EC_{50} reproduction was higher than that for survival and in two other tests the EC_{50s} for both endpoints were nearly the same.

Relationship between soil properties and main endpoints

- *Control*

A Redundancy Analysis (RDA) (CANOCO, 2002) of enchytraeids data showed a strong correlation between species parameters and soil properties for the first two axes (0.962 and 0.515 respectively). Species parameters alone explained 75.1% and 2.8% of the total variance associated with the first and second axis respectively) whilst the interaction between species parameters and soil properties accounted for an additional 19.8% and 0.7% of the total variability, respectively.

The two species (juveniles and adults) are grouped along the first axis in association with higher values of pH (Figure 5). Conditional effects were significant for pH (λ = 0.56; F = 20.2; p = 0.002) and sand (λ = 0.08; F = 3.71; p = 0.028).

Stepwise multiple regression analysis (SPSS, 2003) showed that the survival of adults and the number of juveniles is significantly related with pH (positive effect) for both species (see Model in Tab. 3). Moreover, ANCOVA analysis (Zar, 1996) showed that regression lines between species are coincidental for adults ($F_{2, 32}$ = 1.602; $p<0.05$) and juveniles ($F_{2, 32}$ = 0.005; $p<0.05$).

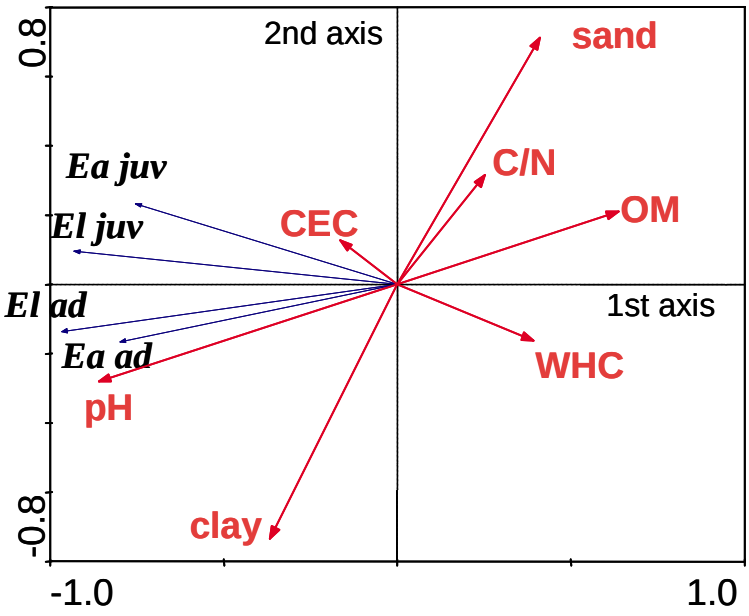

Figure 5: RDA biplot of species and soil parameters (Ea = *Enchytraeus albidus*; El = *Enchytraeus luxuriosus*; ad = adults; juv = juveniles).

Table 3: Stepwise regression models for the test species Ea= *Enchytraeus albidus*, El= *Enchytraeus luxuriosus*, (ad= adults; juv= juveniles;) with the chemical substance (Bet= Betanal) and without and its relation with the soil parameters. Bioassay endpoints and environmental parameters, except pH, were log-transformed. Logarithms of X+1 were applied to bioassay endpoints.

Model	$r^2_{adjusted}$	F	n	p < 0.05
Log (**Ea_ad +1**) = 0.01+0.154***pH (3.2>pH<7.4)**	0.432	12.409	15	0.003
Log (**Ea_juv+1**) = -1.060+0.36***pH (3.2>pH<7.4)**	0.326	8.270	15	0.012
Log (**El_ad+1**) = -0.559+0.236***pH (3.2>pH<7.4)**	0.655	29.470	15	0.000
Log (**El_juv+1**) = -1.071+0.355***pH (3.2>pH<7.4)**	0.478	14.737	15	0.002
Log(**Ea_Bet_EC_{50}juv**)=5.168-2.346***log(WHC)**+0.532***log(OM)** **(42.6>WHC<90); (2.7>OM<8.0)**	0.924	31.393	6	$p_{log(WHC)}$=0.007 $p_{log(OM)}$=0.034

- *Contaminated*

A RDA with *E. albidus* endpoints (NOEC and EC_{50} for adults and juveniles) showed high correlation (1.0) between toxicity endpoints and soil properties for the first two axes. The first and second axis explained 54.7% and 44.2% of the total variability, respectively (Figure 6). Adults and juveniles are separate along the first and the second axis, although this separation is clearer along the second axis. Adults (NOEC

and EC_{50}) are associated with higher values of pH (first axis) whilst juvenile EC50s are closely associated with higher values of sand and juvenile NOECs are associated with higher values of C/N. No significant conditional effects were found.

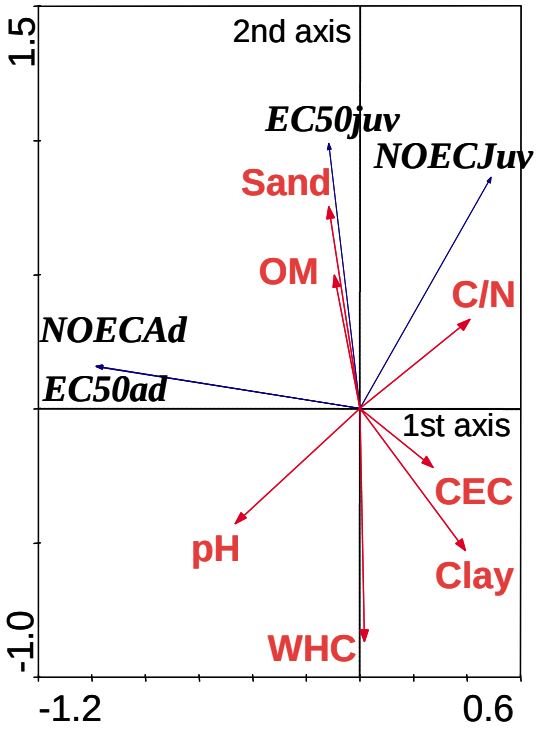

Figure 6: RDA diplot of species (*Enchytraeus albidus*) endpoints and soil parameters.

Stepwise multiple regression analysis (SPSS, 2003) showed that juvenile EC_{50} values are significantly related with Log(WHC) (negative effect) and Log(OM) (positive effect) (see Model in Tab. 3). No statistically significant model could be derived for adult EC_{50} or NOEC (adults and juveniles) values.

A RDA with *E. luxuriosus* endpoints (NOEC and EC50 for adults and juveniles) showed high correlation (1.0) between toxicity endpoints and soil properties for the first two axes. The first and second axis explained 49.4% and 37.2% of the total variability, respectively (Figure 7). This first axis separates adults from juveniles along a gradient of CEC and WHC (lower values are closely associated with juvenile's endpoints). No significant conditional effects were found.

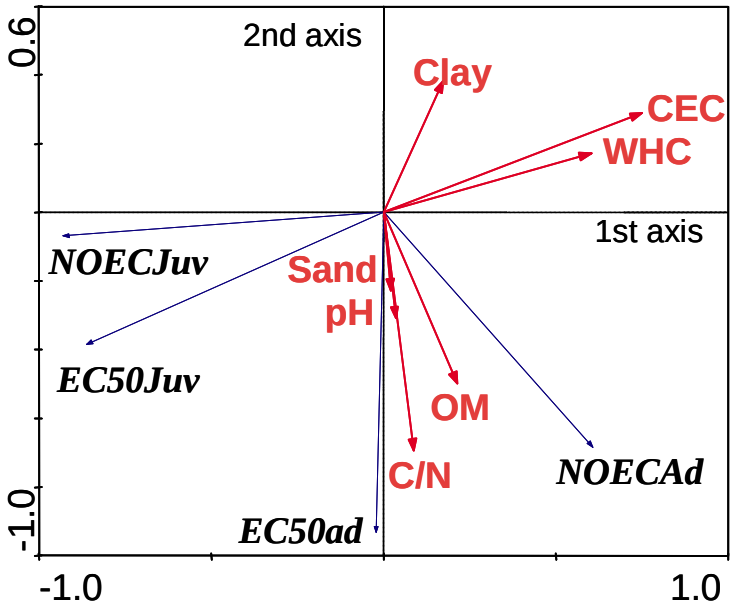

Figure 7: RDA diplot of species (*Enchytraeus luxuriosus*) endpoints and soil parameters.

4. Discussion

First set: Control experiment

In both enchytraeid species, reproduction was shown to be a more sensitive endpoint than mortality. In various cases, (e.g. Nat1 in *E. albidus* or ES1 in *E. luxuriosus*), reproduction was severely affected although survival was not impacted at all.

The preferences of both species for certain soils were neither the same nor completely different. However, it is difficult to identify which soil properties were responsible for the different behaviour.

Additionally, we should not forget that the original EURO-Soils were finely sieved which might have contributed to the sometimes low reproduction, especially of *E. luxuriosus*.

Results from the RDA showed a separation between juveniles and adults reflecting a separation of effects: survival and reproduction. Both species were associated with

higher values of pH, and are sensitive towards pH decreases. This was confirmed in the stepwise models developed: survival of adults and production of juveniles was significantly related with pH in both species. In all acidic soils with a pH lower than 5 (i.e. mainly those being similar to ES5 and ES7) no reproduction occurred and in some (but not all) survival was also impeded. If pH is below 5 survival and reproduction are highly affected; however, apparently very small increases after the value of pH 5 are leading to immediate changes in their tolerance and allowing survival (e.g. ES1) and reproduction (e.g. ES3), hence the pH is no longer exerting its toxic effect. One should consider the hypothesis that pH can have an indirect deleterious effect on the number of juveniles, as a result of decreased adult survival. The effect of pH was already mentioned by Amorim *et al.* (1999) where no reproduction was possible in OECD soil at a pH of 4.4. Moreover, regression lines between species were coincidental for adults and juveniles meaning that the two test species, *E. albidus* and *E. luxuriosus*, are very similar in terms of tolerances towards different soils.

Other soil properties are also influential in terms whether the soil medium is acceptable or not acceptable for the enchytraeids; for instance, reproduction is possible in ES1 (pH = 5.1) and not possible in Nat1 (pH = 6.2). Apparently, organisms were associated with higher values of sand content, maybe due to the fact that a more porous structure of the soil is achieved contributing positively to these soft bodied organisms mobility and gaseous exchanges. The lack of conclusive answers concerning the influence of soil properties other than pH (and sand) indicates that the interactions of several soil properties are responsible for these results. In any case, it is clear that the two test species *E. albidus* and *E. luxuriosus* are sensitive in terms of lethal and reproduction effects to different soil properties.
Finally, as a rough indication from our results, the two enchytraeid species can be tested in soils within the following ranges for:

- pH: 5.1 - 7.4 (recommendation > 5);
- OM content: 2.5 - 8.0 %;
- Clay: 6 - 26 % (one soil with 75 % is not considered here since no values between 26 and 75 % are available);

- Sand: 4 – 80 %;

Soils falling into these ranges are not always fine for the worms, but the respective reason for this failure has to be determined on a case-by-case basis.

Chemical testing

Enchytraeids showed considerable differences in their sensitivity towards Phenmedipham in the different test soils. Using the more ecologically relevant and robust effect value for reproduction, i.e. the EC_{50s}, it was observed that the respective test soil can change these values by a factor of four in *E. albidus* and by a factor of nine in *E. luxuriosus* (in the case of mortality, the range is even higher: 3 – 30 when taking both species together). These changes are considered to be important when using the test results in environmental risk assessment since they are in the same order of magnitude as the assessment factors used when calculating the PNEC from chronic test results.

In the literature, no data are available on the effects of Phenmedipham on either *E. albidus* or *E. luxuriosus*. However, a comparison with data gained in tests with *E. crypticus* is possible; in particular, when the same test design and LUFA St. 2.2 soil have been used as here (Achazi *et al.* 2000, Römbke *et al.* 2000, Hund-Rinke *et al.* 2002). According to these sources, LC_{50} values of 78.9 – 106.9 mg a.i./kg and EC_{50} values of about 33.4 mg a.i./kg were found. These values are in good agreement with the values determined in this study: Referring to the endpoint mortality, *E. albidus* and *E. luxuriosus* seem to be more sensitive than *E. crypticus*, while the effects on reproduction are very similar for all three species (less than 10% difference).

The differences observed in terms of toxicity are probably mainly due to an additional and/or synergistic effect of the different soil parameters. From the RDA for both test species, one can observe a separation between adults and juveniles reflecting the separation of effects at different levels: survival and reproduction. In *E. albidus,* adults (NOEC and EC_{50}) are associated with higher values of pH (ranging from 5.1 to 7.4) whilst juveniles are closely associated with higher values of sand and C/N. Lower toxicity in adults was in close association with higher values of pH. This means that apparently the adults were more sensitive to lower pHs in

comparison to the other soil parameters. While this relationship has been observed beforehand (e.g. Didden & Römbke 2001) to our knowledge it was quantified only in this study.

Lower juvenile toxicity values were associated with higher values of C/N (ranging from 7.7 to 40) and sand (ranging from 3 to 77%): higher microbial activity due to higher C/N values might support a quicker metabolisation of the test substance into a less toxic compound. It is known that the degradation of Phenmedipham in soil starts with a microbially-induced hydrolisation (Domsch, 1992). The association with higher sand contents is hard to understand, despite the fact that, as mentioned before, it could be related with the achievement of a more porous structure of the soil thus contributing positively to these soft bodied organisms' mobility and gaseous exchanges. On the contrary, it would be expected that the higher amount of small particles such as the clay content, due to its adsorptive capacity, would diminish chemical availability (e.g. Baker *et al.* 2003) and therefore the toxicity.

Despite these associations, only juvenile EC_{50} values are significantly related with Log(WHC) and Log(OM): lower WHC and higher OM values are associated with a decrease in toxicity. Relatively to the maximum water holding capacity, higher values are responsible for the improvement of the maintenance of humidity. Therefore the chemical substance is mostly in the water phase, thus more bioavailable for these soft body organisms which in turn increases the toxicity. The adsorptive capacity of OM for organic substances is commonly referenced (e.g., Belfroid and Sijm, 1998): higher OM values are related with less toxicity for the organisms due to the higher adsorption of the organic chemical to these particles and a decrease of the bioavailable fraction.

For *E. luxuriosus*, the RDA shows that lower toxicity in juveniles was associated with lower values of CEC and WHC. Additionally to the possible effect described above, caused by the lower WHC, CEC (ranging from 11.2 to 75.8 mval/100g) seems to have an important influence. According to some authors (e.g. Lock and Janssen, 2001), CEC is a better parameter to estimate bioavailability and ecotoxicity in terrestrial toxicity (but with metals) because this parameter is a measure of the

amount of available sorption sites and thus incorporates the clay, metal oxyhydroxides as well as organic matter of a soil. This means that lower toxicity could be expected in the presence of higher CEC values. Apparently this is a contradictory result but related to metals toxicity and not to organic compounds. In a study done by Peijenburg *et al.* (1999) *E. crypticus* was tested in 20 Dutch field soils contaminated with Cd, Cu, Pb and Zn. Multivariate expressions that describe uptake rate constants and bioaccumulation factors as a function of soil characteristics were derived. pH and CEC were the most important parameters, but these were differing in their relative importance with each metal: pH and clay content in the case of Cd were the dominant soil properties determining availability and bioavailability, Al_{ox} and pH for Zn, whereas for Pb it is pH, clay, CEC, Fe_{ox} and OM. Obviously, the experiences gained about the interactions between soil properties and heavy metals cannot be extrapolated to other chemicals like the organic Phenmedipham.

Additionally, the OECD artificial soil, the most commonly used test substrate in soil ecotoxicology, is among the soils responsible for higher survival and reproductive rates in comparison with a broad range of natural soils. Despite that the results reported here are with just one test substance, this issue calls for attention to the further use of this substrate and urges for a re-evaluation of the OECD artificial soil. Interestingly, in the tests with enchytraeids the toxicity of Phenmedipham in OECD artificial soil was not always lower than in field soils, while in similar tests with collembolans the sensitivity of this pesticide was always higher in field soils (Amorim et al. 2004). However, it seems to be appropriate to reduce the peat content of OECD artificial soil (e.g. from 10 to 5 %) in order to enhance its field relevance.

5. Conclusions

From this study one can conclude that soil properties limit the use of test species: enchytraeids showed high sensitivity to changes in soil parameters and were mainly affected by low pH. Therefore, soil type is an important issue when discussing which species can be used for risk assessment and which effects can be expected due to

extrapolation to the biotic community. More data using more soils and species are required to understand the effect of soil properties in soil toxicology. Nevertheless, it was clear that certain soil properties such as OM and WHC or pH, CEC, C/N and clay content did interact with the chemical and the organisms.

In the present study, EC_{50}s in enchytraeids changed by a factor of 9 for juveniles and nearly 30 for the adults of *E. luxuriosus* (maximum values; slightly lower values were found for *E. albidus*), which shows how important the test soil can become for the environmental risk assessment of chemicals. In this context, the further usage of OECD artificial soil needs also to be discussed.

6. Acknowledgements

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Chapter 6

Effects of different soil types on the Collembolans *Folsomia candida* and *Hypogastrura assimilis* using the herbicide Phenmedipham

6. Effects of different soil types on the Collembolans *Folsomia candida* and *Hypogastrura assimilis* using the herbicide Phenmedipham

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ABSTRACT

Soil ecotoxicology studies are usually performed in standard soils, such as OECD artificial soil or LUFA ST. 2.2, a natural soil. When assessing the toxic effects in the environment, soil properties are often different from those in standard soils, which might lead to a different exposure situation for the test species and, therefore, to misleading conclusions. Selected to cover a broad range of properties and based on the Euro Soils concept, 17 different soils were studied regarding their suitability to two test species: *Folsomia candida* and *Hypogastrura assimilis* (Collembola). In reproduction tests, the test species reacted differently to the soils. *F. candida* is less affected by soil properties: in untreated soils (i.e. controls) between 500 and 1200 juveniles/vessel were found. These differences can be attributed to normal interindividual variability. *H. assimilis* showed a significant correlation with WHCmax and also a tendency to lower the reproductive output in soils with a low pH (< 4). Therefore, some soils revealed to be inappropriate for tests with *H. assimilis*. In the main tests, the effect of the reference test substance Phenmedipham (formulation Betosyp) was studied in those soils where sufficient reproduction was determined beforehand. Clearly, the chronic endpoint was more sensitive than survival when testing Phenmedipham. In *H. assimilis*, due to high variability and low effects in the tested dosages, no conclusions could be drawn. In *F. candida*, different soils caused different toxic effects: juveniles preferred soils with high C/N ratio. Higher microbial activity might support a quicker metabolisation of the test substance. In general, the toxic response is caused by a synergistic action of several soil properties, each of them exerting a too small of effect to be clarified with the available set of data.

Keywords: soil types, SIM-Soils, EURO-Soils

1. Introduction

In toxicity testing the standardization of methods is required due to reasons of comparability and quality assurance. Most ecotoxicological soil tests are performed with two different standard soils, either an artificial soil (OECD, 1984) or the natural LUFA 2.2 soil (Schinkel 1985). However, the toxicity of chemicals may be affected by properties of the soil in which the chemical is tested. This problem can be addressed in two ways: either as many soils as practically feasible can be tested (Jepson et al., 1994) or a unifying concept is developed (Van Gestel, 1997). Despite the fact that many complex processes occurring in soil are at least partially known (Gawlik et al., 2003b), the shear number of different soils with their many combinations of main soil properties complicates the definition of such a unifying concept. Therefore, the problem is often ignored. For example, approaches to establish threshold values for organic xenobiotics or heavy metals, e.g. in the context of the sludge amendment, do not consider sufficiently the influence of general soil parameters on the mobility, availability and ecotoxicity of the compounds being regulated (Langenkamp and Marmo, 2001).

For these reasons, a set of reference soils – the so-called EURO-Soils – was introduced in 1990 to create a common basis for a better comparison and quality control of soil sorption data (Kuhnt et al., 1994a). Firstly five, finally six regionally representative soils were identified, collected, prepared and characterised as reference soils for chemical testing in the EU (Gawlik et al., 1996). The demand for EURO-Soils grew dramatically after their introduction, and it became quickly evident that the original EURO-Soils are not a suitable source for ecotoxicological standard tests due to the fact that the available amount is simply not sufficient.

Recently, Römbke and Amorim (2004) suggested that in fact each soil similar in terms of their main properties (i.e. texture, pH, C/N ratio and organic matter content) to one of the six EURO-Soils could be used for ecotoxicological tests To validate this hypothesis, such tests were performed in the original Euro Soils, similar soils (SIM-Soils) and the standard OECD and LUFA 2.2 soils for comparison purposes. While

several species were chosen as test organisms, here we present the results with the two collembolans *Folsomia candida* and *Hypogastrura assimilis*. They were selected as test species because of their easiness of culture and high reproductive rates. An international test guideline exists for the first species (ISO 1998), but a standard procedure has been proposed for the latter (Folker-Hansen *et al.*, 1996). *H. assimilis* differs from *F. candida* mainly in its way of reproduction: the former reproduces sexually, the later is a parthenogenetic species. In addition, *H. assimilis* shows some social behaviour, which has not been observed in *Folsomia* (or onychiurid species) (personal communication by Paul Henning Krogh). Reproduction tests were performed with both species in the several soils. EC_{50} as well as NOEC values were calculated and the influence of the respective soil properties on the test results was statistically evaluated. Thus, in this work we aimed to:

- Assess whether the two collembolans are suitable as test species;
- Determine in which soils the collembolans can be tested;
- Investigate what is the influence of the soil properties on the test results.

2. Materials and Methods

Test Species

Folsomia candida Willem 1902 (Collembola: Isotomidae) is a blind, unpigmented euedaphic collembolan reproducing parthenogenetically (Hopkin 1997). *Hypogastrura assimilis* Krausbauer (Collembola: Poduridae) is an epi- to hemiedaphic species, pigmented and with eyespots. It is a sexually reproducing species. The females have approximately twice the size of males and are lighter coloured (Folker-Hansen *et al.*, 1996). Both species are easily cultured in laboratory in a moistened substrate of plaster of Paris/charcoal (mixture 8:1) prepared according to Usher & Stoneman (1977). Organisms are maintained in laboratory at 20ºC, in the dark, and fed dried backer's yeast (*Saccharomyces cerevisae*).

Test Substance

The test substance Phenmedipham, an herbicide, was applied as the formulation Betosyp (formerly known as Betanal; STÄHLER AGROCHEMIE, 157g/l a.i.) to the soils in the following concentrations: 0.1, 0.32, 1, 3.2 and 10 mg a.i./kg soil DW. In the case of the OECD soil, the concentrations were 1, 3.2, 10, 32 and 100 mg a.i./kg soil DW. These concentrations were based on the results of a Range-Finder test, where no effects occurred up to 10 mg a.i./kg. In the case of the LUFA 2.2 soil, the concentrations were 5, 8.75, 12.5, 16.25 and 20 mg/kg soil DW, based on the results of a ring-test (Hund-Rink *et al.* 2002a). The contamination of all test substrates was done by mixing an aqueous solution of the test chemical into the pre-moistened soils, separately for each test concentration. After homogeneous mixing, sub-samples of soil were introduced into the individual test vessels.

Test procedures

Synchronized cultures were established for the experiments by removing egg clusters from stock cultures into new culture vessels. Two days after the start of hatching, juveniles were transferred into a new vessel, where they were fed and watered. After approximately 10 days for *F. candida* and 16 days for *H. assimilis* the organisms were in the correct life stage to start the test.

Test procedures were as described in the ISO guideline 11267 for *F. candida*. Ten organisms 10 - 12 days old were placed in each test vessel, already containing the pre-moistened test soil and the food supply. The vessels were covered with a parafilm layer in which a few holes for airing were made. Food and water was replenished weekly. After four weeks the test ended and each test vessel was filled with distilled water which was gently mixed with a spatula. Afterwards, juveniles and adults were floating on the surface. Due to the addition of a few drops of dark ink a higher contrast between the white organisms and the black background was obtained. A digital photograph of the water surface plus collembolans was made. The collembolans on the image could later be easily counted using computer software SigmaScan Pro 5. Some replicates are randomly selected and the pictures are

checked by hand, in order to validate the accuracy of the program. Adults and juveniles were easily distinguished by their size.

The test procedure for *H. assimilis* was similar, except that the organisms were 16 - 19 days old at the start of the test to distinguish between males and females. Ten males plus ten females were introduced per test vessel. The test duration was 3 weeks. *H. assimilis* forms clusters when floating on the water surface, so counting of individuals by the software is not possible. To individualise the organisms the end of the test differs: test vessels were covered with a narrow net and connected to an empty vessel by a double lid. Afterwards, the vessels were inverted, covered with black paper and placed under a heating source for 24 hours. Since the organisms tended to escape from the heat in the upper vessel they fell into the empty vessel at the bottom. Finally, the organisms were immobilized with alcohol, digital photos were made and the same software package was used for counting. Adults and juveniles were not distinguishable from each other.

Test soils

The main properties of the test soils (pH, Organic Matter, Carbon/Nitrogen, Cation Exchange Capacity, maximum Water Holding Capacity, clay, silt and sand content) are given in Table 1. Their selection is described in detail by Römbke and Amorim (2004).

Artificial soil (OECD, 1984) is constituted by 69% sand, 20% kaolin clay, 10% sphagnum peat and 0.3 to 1% of $CaCO_3$ for pH adjustment (6±0.5). LUFA 2.2 is a natural standard soil from Speyer (Germany). The codes given for the natural soils are translated as follows: ES means EURO-Soil; ESo means that the soil is a sample from the same site as the original ES; the numbers mean that the soil is similar to a certain ES number (X = the soil could not classified to a certain ES class) and the other codes represent the first three letters of the soil original place name: Nat1: Natzungen; Hoh2: Hohenlimburg; Coi2: Coimbra; Sch3: Schmallenberg; Mon4: Mönninghausen; Tau4: Taubenheide; KarX: Karlsruhe (Schluttenbach); Ren7: Gladbeck-Rentfort. At least one natural soil from each class was tested. Not surprisingly, it was most easy to find soils belonging to ES class 3, since this class

represents “normal” agricultural soils in Central Europe. In some cases, in particular the original EURO-Soils, the amount of available soil was so small that not all tests could be performed. With the exception of soil ES7, bought from the University of Vienna (Austria), the other samples were from the European Chemical Bureau (Ispra, Italy), where only a small amount remained available.

Table 1: Main characteristics of the tested soils and the relative EuroSoils (ES) properties: pH, OM, C/N, grain size distribution, CEC and WHC.

SOIL	pH ($CaCl_2$)	O.M. (%)	C/N	Clay (%)	Silt (%)	Sand (%)	CEC (mval/100g)	WHC (%)
ES1	5.1	2.7	7.7	75	22	3	29.9	62.6
Nat1	6.2	1.7	8.7	33	66	5	40.7	58.4
ES2	7.4	6.4	18.5	23	64	13	28.3	68,5
Hoh2	6.2	12.9	25.0	6	61	33	78.3	73.9
ES3	5.2	6.5	13.3	17	37	46	18.3	42.6
ESo3	5.2	6.0	11.8	18	38	44	74.5	-
Sch3	5.4	4.1	10.4	23	45	32	68.5	67.4
Coi3	6.7	6.5	17.0	26	60	14	75.8	68.1
LUFA 2.2	5.8	4.4	14.0	6	17	77	11.2	55
ES4	6.5	2.9	9.7	20	76	4	17.5	42.9
Mon4	6.5	2.5	9.7	11	77	12	20.7	53.2
Tau4	6.9	2.9	9.7	17	79	4	61.3	63.1
ES5	3.2	15.9	30.8	6	13	81	32.7	38.7
Eso5	3.2	9.2	29.7	10	12	79	87.0	100.1
ES7	4.4	11.5	14.2	19	35	46	5.0	80.6
Ren7	3.8	8.7	11.0	18	40	42	132	121.8
ESoX	6.3	8.9	23.5	31	33	36	-	64.0
KarX	3.6	10.6	45.9	13	58	29	173	71.9
OECD artificial	6.0	8.0	Ca. 40	10	10	80	45.8	Ca. 90

Experimental setup

The whole study was done in two parts: In the first set of experiments the survival and reproduction of the two species was tested in all soils in a control situation, i.e. without any contamination. The aim was to verify the suitability of the individual

soils for a certain species. In the second set of experiments, only the soils where reproduction was within the validity range (mortality < 20% and number of juveniles > 100 per test vessel) as defined in the ISO guideline (1998) were tested with the test substance Phenmedipham.

Statistical procedures

There are two main hypothesis to be tested. It is hypothesised that the measured soil properties influence:

1) The survival and reproduction of the test organisms (tests without chemicals);
2) The toxicity of the toxic compound, either by directly altering the exposure (e.g. due to different adsorption and bioavailability) or by adding an extra stress factor for the organisms (i.e. in addition to the chemical).

Redundancy Analysis (RDA) was applied to the results of survival and reproduction of enchytraeids and collembolans maintained in different soil types in the absence of toxicants. The analysis was performed with Canoco for Windows 4.5 (Ter Braak and Smilauer, 2002) using survival and reproduction of each species to play the role of species, and the physical and chemical parameters of each soil to play the role of environmental data. All data was log-transformed prior to the analysis, except for pH values. Additionally only the two extreme categories (sand and clay, excluding silt) of the three texture classes due to the interdependence of the individual parameters were used. Species data was centered and normalised within Canoco for Windows. A similar procedure was used to analyse toxicity data from exposure of collembolans in different soil types. However, in this case toxicity parameters (EC_{50} and NOEC) were used to play the role of species. Conditional effects of environmental data on species data were assessed using Monte Carlo permutation tests with automatic variable selection from within Canoco for Windows.

Stepwise Multiple Regression models were developed, using the statistical software package SPSS 12.0 (SPSS, 2003), to quantify the relationship of the biological data with soil data. All but the pH data was also normalised using logarithms (X+1) and silt was excluded.

Analysis of variance and Bivariate Spearman Correlations were calculated using SPSS 12.0 (SPSS, 2003). EC_{50s} and NOECs were calculated using the ToxRatPro program (ToxRat, 2003).

3. Results

First set: control experiments

In total, the mortality and reproduction of *F. candida* was tested in 17 soils (Fig. 1). Some animals died in all soils, however in nearly all cases the mortality was ≤10%. Only in three of the original EURO-Soils (ES1, ES3 and ES4) up to 20% mortality occurred. The number of juveniles varied by a factor of about 2 (minimum: about 450 in ES4; maximum: about 1000 in LUFA St. 2.2), but in any case was at least four times higher than the validity criterion as defined by the ISO guideline. No statistical differences could be determined between OECD soil (reference) and the other soils in terms of the number of juveniles (One way ANOVA, Dunnetts' two sided; $p>0.05$).

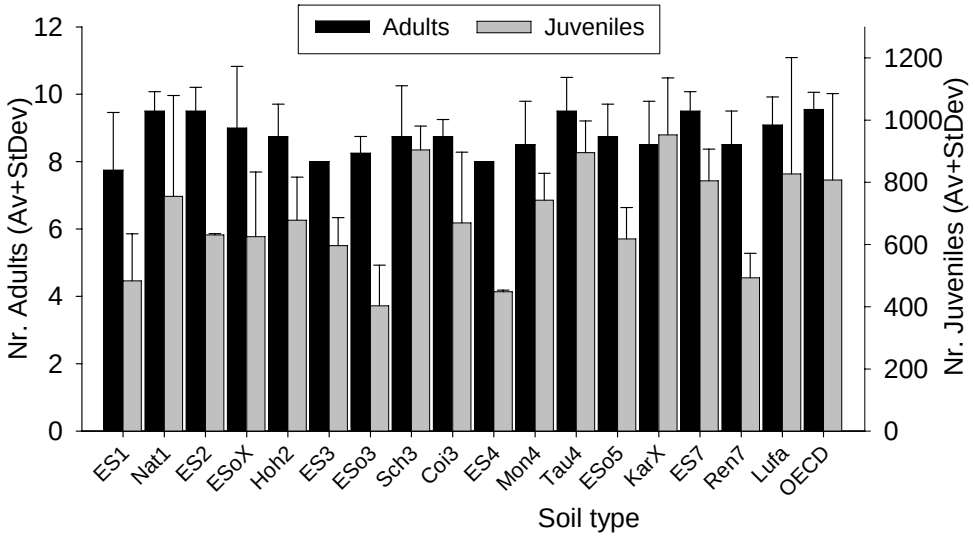


Figure 1: Results obtained exposing *Folsomia candida* to different soil types (OECD soil, LUFA2.2, Euro-Soil and similar soils). Graph shows the average number + standard deviation.

The second collembolan species, *H. assimilis*, could only be tested in 11 soils (the amount of the original EURO-Soils was too small). The results differed completely from the tests with *F. candida*. The number of individuals in the various soils was highly variable (Fig. 2) and fluctuated between 0 (ESo5) and 350 (ESoX)) individuals per test vessel. In the soil Ren7 adults survived but no reproduction was possible. The results in these soils were also statistically significantly different from the results in the OECD soil (One way ANOVA, Dunnetts' two sided; $p<0.05$).

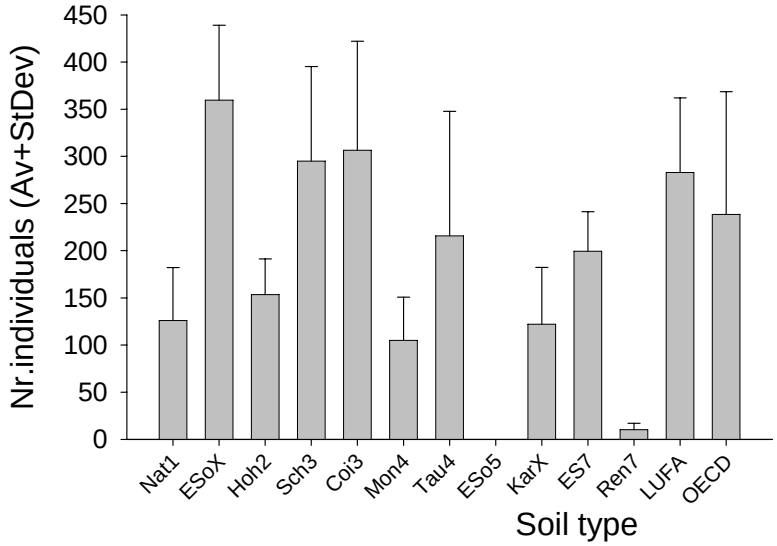


Figure 2: Results obtained exposing *Hypogastrura assimilis* to different soil types (OECD soil, LUFA 2.2, Euro-Soil and similar soils). Graph shows the average number + standard deviation.

The variability of the results for *H. assimilis* is clearly higher and the species reacts more sensitively to soil properties than *F. candida*. In addition, minimum and maximum average numbers of juveniles obtained were considerably different for each species: between a minimum of 493 and maximum of 953 for *F. candida*, and a minimum of zero and a maximum of 359 for *H. assimilis*.

The RDA with collembolans data showed a high correlation between species parameters and soil properties for the first two axes (0.657 and 0.544 respectively). Species parameters alone explained 33.1% and 3.7% of the total variance associated with the first and second axis, respectively, whilst the interaction between species

parameters and soil properties accounted for an additional 54.4% and 6.1% of the total variability.

Species were grouped along the first axis in association with higher values of WHC and C/N (Figure 3). Juveniles and adults of *F. candida* are separated along the second axis in association with a gradient of WHC and CEC (higher values are associated with juveniles) and pH (higher values are associated with adults). No significant conditional effects were found.

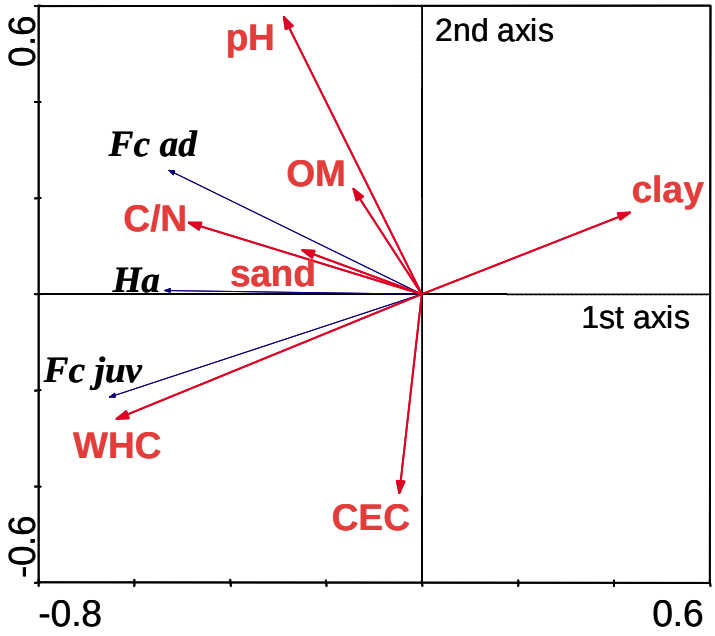


Figure 3: RDA biplot of species and soil parameters (Fc = *Folsomia candida*; Ha = *Hypogastrura assimilis*; ad = adults; juv = juveniles).

Stepwise multiple regression analysis showed significant relationship between the number of individuals of *H. assimilis* at the end of the test and the Log(WHC) (negative effect) (see stepwise model in Tab.3). A negative correlation between individual numbers and pH was found, although pH was not included in the final regression model for *H. assimilis*.

Second set: experiments with Phenmedipham

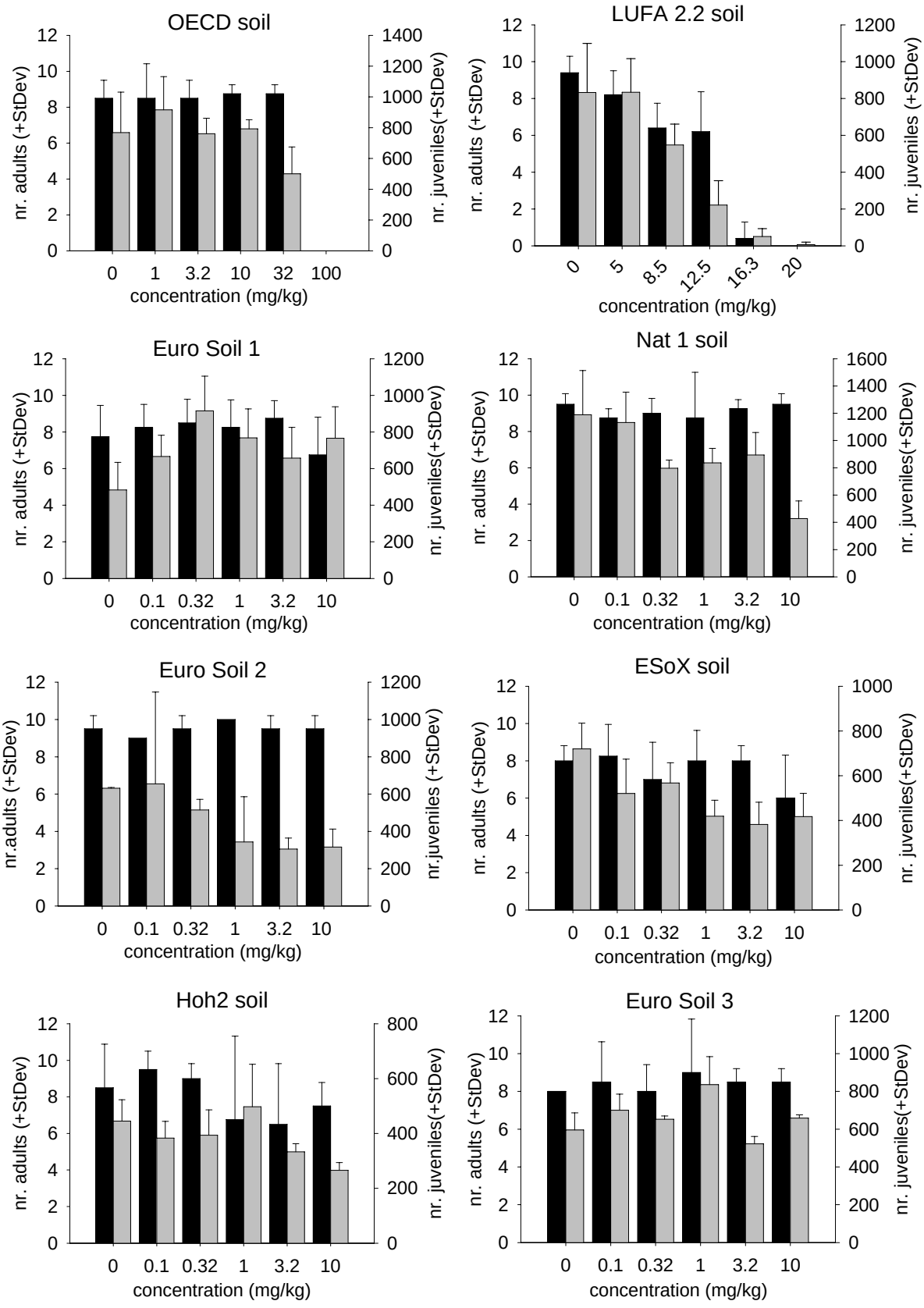
In the case of *F. candida*, all soils tested as control were tested with Phenmedipham (as Betosyp), and results can be seen in Figure 4. In the case of *H. assimilis* ESo5

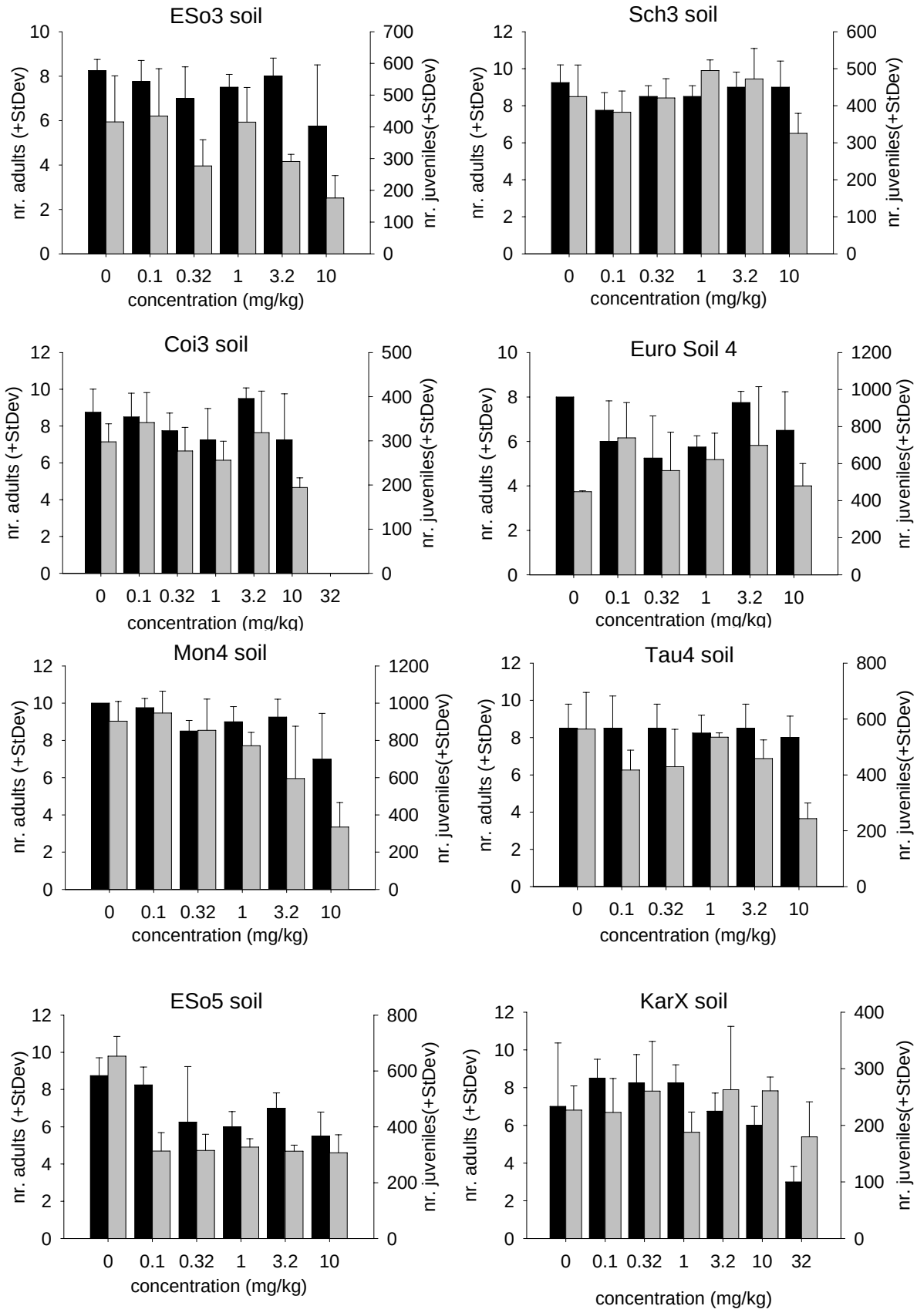
and Ren7 showed none or nearly no reproduction in the control test run, hence all but these two soils were used as test substrates. The test results with *H. assimilis* were very variable (among replicates and between treatments) and without any dose-response relationship. Due to this situation no effect levels (EC_{50}s or NOECs) could be calculated. In the controls, a higher range concerning the number of organisms was found than in the first tests without contamination. This is caused by the differences found in the soil ESoX, where a number of 990 (±252.2) individuals was found – nearly three times the maximum observed before.

The effect values of the tests with *F. candida* are summarised in Table 2.

Table 2: Resulting EC_{50}s and NOECs from the exposure of *Folsomia candida* to Phenmedipham (a.i.). (n.d. = not determined due to mathematical reasons).

Soils	*Folsomia candida*			
	EC_{50}		**NOEC**	
	Survival	**Reproduction**	**Survival**	**Reproduction**
ES1	>10	>10	≥10	≥10
Nat1	>10	6.8	≥10	0.1
ES2	>10	4.4	≥10	0.32
Hoh2	>10	22.8	≥10	3.2
ES3	>10	>10	≥10	≥10
ESo3	>10	9.4	≥10	3.2
Sch3	>10	>10	≥10	3.2
Coi3	>10	12.2	≥10	3.2
LUFA 2.2	10.6	10.1	<5	5.0
ES4	>10	>10	≥10	≥10
Mon4	>10	6.0	≥10	1.0
Tau4	>10	8.3	≥10	3.2
ESo5	n.d.	n.d.	0.1	<0.1
ES7	>10	7.9	≥10	<0.1
Ren7	>10	4.5	≥10	0.32
ESoX	>10	n.d.	≥10	<0.1
KarX	>10	>10	10	≥32
OECD	51.9	39.2	32	10


OECD soil
nr. adults (+StDev)
nr. juveniles(+StDev)
0 1 3.2 10 32 100
concentration (mg/kg)
LUFA 2.2 soil
nr. adults (+StDev)
nr. juveniles (+StDev)
0 5 8.5 12.5 16.3 20
concentration (mg/kg)
Euro Soil 1
nr. adults (+StDev)
nr. juveniles(+StDev)
0 0.1 0.32 1 3.2 10
concentration (mg/kg)
Nat 1 soil
nr. adults (+StDev)
nr. juveniles(+StDev)
0 0.1 0.32 1 3.2 10
concentration (mg/kg)
Euro Soil 2
nr.adults (+StDev)
nr.juveniles (+StDev)
0 0.1 0.32 1 3.2 10
concentration (mg/kg)
ESoX soil
nr. adults (+StDev)
nr. juveniles(+StDev)
0 0.1 0.32 1 3.2 10
concentration (mg/kg)
Hoh2 soil
nr. adults (+StDev)
nr. juveniles(+StDev)
0 0.1 0.32 1 3.2 10
concentration (mg/kg)
Euro Soil 3
nr. adults (+StDev)
nr. juveniles(+StDev)
0 0.1 0.32 1 3.2 10
concentration (mg/kg)


ESo3 soil
Sch3 soil
Coi3 soil
Euro Soil 4
Mon4 soil
Tau4 soil
ESo5 soil
KarX soil
nr. adults (+StDev)
nr. juveniles(+StDev)
concentration (mg/kg)
0 0.1 0.32 1 3.2 10
0 0.1 0.32 1 3.2 10 32

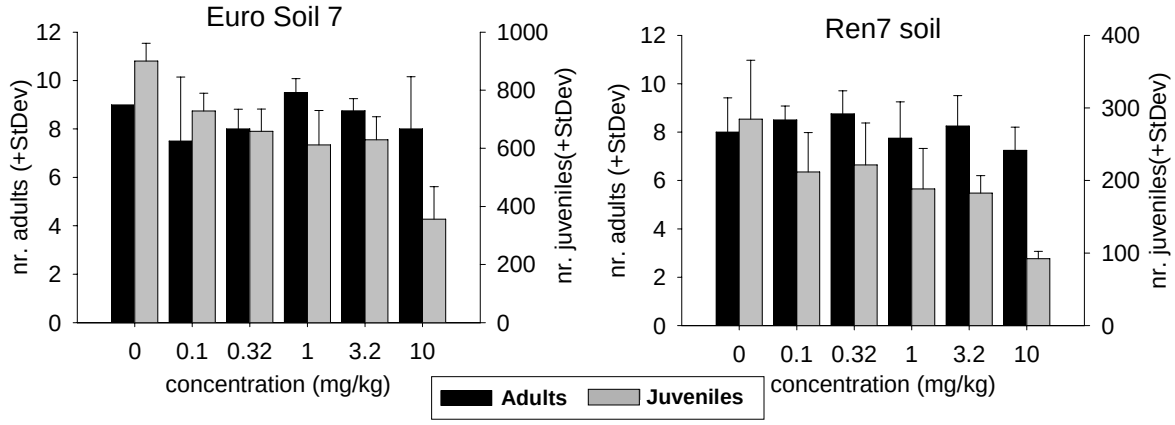


Figure 4: Results obtained by exposing *F. candida* in different soils to Phenmedipham (Average numbers).

Due to the fact that the concentrations were chosen in a way that the main endpoint reproduction (= number of juveniles) could be determined, it was hypothesised that the effect values for adults (EC_{50} and NOEC) are usually higher than the highest test concentration (10 mg a.i./kg). The only exceptions are the two acidic soils ESo5 and KarX, where the NOECs were determined as 0.1 and 10 mg a.i./kg. It should be noted that the concentration range for the two standard soils was chosen so that the effects on mortality could be also determined. While in the artificial soil both effect values are relatively high (51.9 and 32 mg a.i./kg, respectively) they are clearly lower in the LUFA St. 2.2 soil (10.6 and <5 mg a.i./kg, respectively).

The effect values for the juveniles varied between 4.4 and 39.2 mg a.i./kg (EC_{50}) and <0.1 and ≥ 32 mg a.i./kg (NOEC). So, the EC_{50} values can change by approximately a factor of 10, while the NOECs covered an even broader range, with a factor of > 320. Clearly different results were obtained in the different soils. In addition, there was also a distinction between the different EURO-Soil groups in terms of toxicity. On a first glance, the results in the standard soil LUFA St. 2.2 and, in particular, the acid KarX soil are difficult to understand: In both soils the NOEC values for adult mortality are lower than the NOECs for reproduction. In the case of the LUFA St. 2.2 tests, the effect, given as EC_{50}, on both endpoints is quite similar.

In nearly all cases the control mortality was ≤10%. The number of juveniles in controls of the chemical testing varied by a factor of about 5 (minimum: 226.7 in KarX soil, maximum: 1189.7 in Nat1), but in any case was at least two times higher than the validity criterion as defined by the ISO guideline.

Table 3: Resulting stepwise regression models for the test species Fc= *F.candida* and Ha= *H. assimilis* (juv= juveniles;) with the chemical substance (Bet= Betanal) and without and its relation with the soil parameters. Bioassay endpoints and environmental parameters, except pH, were log-transformed (X+1).

Model	$r^2_{adjusted}$	F	n	p
Log (**Ha**) = 7.186-2.749***log(WHC) (53.2>WHC<100.1)**	0.429	8.510	10	0.017
Log (**Fc_Bet_EC_{50}juv**)= 0.036+0.773***log(C/N) (7.7>C/N<45.9)**	0.629	16.267	11	0.004

An RDA with *F. candida* data could not be conducted since the available dataset had too many undetermined values (e.g. EC_{50} survival > 10; see Tab. 2). Nonetheless, a significant correlation was found between the Log(EC_{50}) for juveniles and the Log(C/N) (positive effect) (see stepwise regression model in Tab. 3)

4. Discussion

First set: control experiments

In the tests with *F. candida*, no major differences were observed in terms of survival and reproduction in the different soils. The validity criteria were always fulfilled. Nevertheless, the juvenile numbers varied in the different soils, meaning that the soil properties somehow might have influenced reproduction. As expected, the endpoint reproduction is more sensitive to soil differences than survival; i.e. the chronic endpoint is more appropriate for this kind of studies.

However, any relationship between changes in the number of juveniles and soil properties must be discussed with caution, since these changes can also be partially attributed in part to normal inter-individual differences. For example, Crouau and

Cazes (2002), found that the variability in juvenile numbers in the standard reproduction test (i.e. performed with artificial soil) with *F. candida* had several causes; among them are the mortality of adults and the variability induced by the use of animals which hatched on three successive days.

Relatively to pH, one could observe that an influence of the pH occurred in some but not all tested soils, since there was a tendency to a lower number of *H. assimilis* individuals at lower pH values. Sandifer & Hopkin (1996) tested *F. candida* in artificial soil at pH values of 6.0, 5.0 and 4.5 in the standard laboratory test. There was no clear relationship between adult survival or juvenile production and soil pH, but an overall decrease in reproduction was observed in the control samples with pH values of 5.0 and 4.5 in comparison to those at pH 6.0. In a similar experiment, Greenslade & Vaughan (2003) studied an even wider range of pH values, finding an optimum of juvenile numbers at pH-values of 5.38 to 6.62. Interestingly, at lower pH values (down to 3.47) the number decreased to about 50% of the optimum number, while at higher pH values (7.65 and 8.03) there was a strong decrease down to zero. The animals seem to be sensitive towards very basic soils. The soils tested in our experiment covered a pH range between 3.2 and 7.4. Basically our results confirm the work of the former authors: there is an influence of the pH on reproduction (especially for *H. assimilis*), but within a range of 3.2 to 7.4 it is not big enough to significantly impede the number of juveniles in *F. candida*.

Stepwise multiple regression analysis only showed a significant relationship between the number of individuals of *H. assimilis* at the end of the test and the Log(WHC) (negative effect). Similar results were observed by Van Gestel & Diepen (1996), who studied the effect of different soil moisture contents on *F. candida* in OECD artificial soil: 25, 35, 45 and 55%, corresponding to 28, 40, 51 and 63% of the WHC. These authors found that the collembolans produced more eggs at lower moisture contents, but these eggs hatched somewhat later than those produced at higher moisture levels. It is not known at which moisture level no eggs are produced anymore, but due to the well-known susceptibility of insects to dryness it must be somewhere close to the

lowest level tested (Edney, 1977; Bursell, 1970). Perhaps there is a similar effect in *H. assimilis*.

Apparently, other soil properties are exerting an effect but no statistically significant relation to any other individual parameter could be observed. From the Redundancy Analysis, although no significant conditional effects were found, one can see that juveniles and adults of *F. candida* are separated along the second axis in association with a gradient of WHC and CEC (associated with juveniles) and pH (associated with adults).
In any case the variability of the results strongly impedes the identification of these probably additive effects.

Second set: experiments with Phenmedipham

Due to the fact that *H. assimilis* showed no consistent effects at the tested concentrations of Phenmedipham and a high variability within treatments, no conclusions can be drawn concerning the interactions between the chemical and the soil properties. This can be due to the sexual mode of reproduction of the species, a factor of increased variability compared to the parthenogenetically reproducing *F. candida*. Additionally, the extraction and counting method for *H. assimilis* was less accurate then the one for *F. candida*, which probably also contributes to the variability in results. Therefore, it is strongly advised to use the improved methodology adopted by Krogh *et al.* (1998) in order to reduce this variability.
Phenmedipham is used as a reference substance according to ISO guideline 11267 (1998), so a broad data set from tests in standard LUFA St. 2.2 is available (Achazi et al. 2000). This substance has also been used as an external control in a ring test sponsored by the German Federal Environmental Foundation (Hund-Rinke et al. 2002b). According to these sources, the LC_{50} values for *F. candida* differ between 14.8 and 15.4 mg a.i./kg, which is very close to the value of 10.6 mg a.i./kg as determined in this study. Actually, effects on reproduction occurred at nearly the same concentration with EC_{50} values between 9.1 and 10.8 mg a.i./kg, in agreement with our results (10.1 mg a.i./kg). Just for reasons of completeness it should be mentioned that the fulfilment of the validity criteria (i.e. mortality of adults and

number of juveniles in the control) were also very similar in the cited studies and in the tests reported here; i.e. the reproducibility of this collembolan test is very good. To assess the general sensitivity of *F. candida* it is interesting to note that in an avoidance test this species was affected by Phenmedipham at concentrations > 3.5 mg a.i./kg, while other Collembolan species were slightly more sensitive, reacting at about 2 mg a.i./kg (Heupel, 2002).

The sensitivity of *F. candida* towards Phenmedipham was different in the various soils. A significant correlation was found between the Log(EC_{50}) for juveniles and the Log(C/N) (positive effect). Juvenile toxicity was related with higher values of C/N (ranging from 7.7 to 40): higher microbial activity might support a quicker metabolisation of the test substance. It is known that the degradation of Phenmedipham in soil starts with a microbially-induced hydrolisation (Domsch, 1992).

Few authors have studied the effect of soil properties on the toxicity of chemicals to *F. candida*. Martikainen (1996) studied the toxicity of dimethoate to *F. candida* in three different soil types (artificial soil, clayey soil, and humus sandy soil). Since its use in the SECOFASE project this insecticide has often been used as a reference substance (Løkke and Van Gestel, 1998). The organic matter content of the soil was negatively correlated with the toxic effects of dimethoate. Phillips *et al.* (2002) investigated the ecotoxicity of the chemical-warfare agent (CWA) HD (Mustard) using *F. candida*. Toxicity tests were conducted using standard artificial soil (SAS; 10% OM; 6 pH), O'Neill-Hall sandy loam (OHSL; natural soil with 4.3% OM; 5.1 pH), and Sassafras sandy loam (SSL; natural soil with 2% OM; 4.9 pH). HD toxicity to both adults and juveniles was greater in SSL than in the two other soils. Smit and Van Gestel (1998) evaluated the influence of soil characteristics and the way of contamination on the bioaccumulation and toxicity of zinc for *F. candida*. In contaminated soils without further treatment, zinc toxicity was related to organic matter and clay content of the soil (for percolated and aged soils the variation in effect concentrations between test soils was reduced). Van Gestel and Mol (2003) studied the effects of cadmium on survival, growth and reproduction of *F. candida* in

four soils differing in organic matter (3.0-10.9%) and clay content (1.4-5.2%) but not in pH (approx. 6.0). EC_{50} values for effects on reproduction ranged between 53.7 and 193 mμg Cd/kg dry soil (after 4 weeks). The absence of a consistent relationship between cadmium toxicity and soil properties suggested that differences of less than a factor of 3 to 4 in organic matter and clay content, in soils with the same pH, do not lead to significant differences in cadmium toxicity to Collembola. Since the OECD artificial soil had the highest content of organic matter and clay, as well as the highest CEC, a very low toxicity would be expected for this soil. This was indeed the case after 4 weeks, as comparable to our own experiments, but at other time intervals toxicity was lower in other soils.

5. Conclusions

The most important result of this study is that important soil properties in a wide range do not limit the use of *F. candida* in ecotoxicological standard tests. Soil had an important influence on *F. candida* when tested with Phenmedipham; i.e. the EC_{50}s of juveniles changed by a factor of approximately 10. Clearly juveniles prefer soils with a high C/N ratio, while their preferences relatively to other soil properties are less clear. More data using more soils and species are required to understand the effect of soil properties in soil ecotoxicology.

H. assimilis showed to be a more sensitive species to different soils than *F. candida*, although the inherent high variability of results in *H. assimilis* and the less feasible and accurate extraction and counting procedures were acting as negative factors. Therefore, this species cannot be recommended for ecotoxicological standard tests unless technical improvements are made. However, the variability caused by the sexual mode of reproduction may be a problem.

The further role of the OECD artificial soil in ecotoxicology should be discussed. Test results with this soil were often those showing the lowest toxicity. Despite that this observation is based on tests with just one chemical, it is clear that the relatively

high organic matter content is the main cause of an often reduced bioavailability of a test substance, and thus its toxicity. For example, a reduction of the peat content (e.g. to 5% peat) would probably be more realistic while still being acceptable for soil invertebrates. In addition, the use of natural peat in a standard substrate should be re-considered, since different kinds of peat are known to induce changes in the fate of chemicals and the behaviour of organisms. Still, the possibility to get well comparable results by using this artificial substrate is worthwhile, but it is simply not representative of the diversity of natural soils (and might underestimate toxicity). However, it is unlikely that the OECD artificial soil will be completely discharged in the future. Instead, it might serve as an external control (probably with a reduced amount of organic matter) in order to secure the quality of the individual test system. The results presented here show that soil toxicity testing should not rely solely on tests with artificial soils, but should include assays with reproductive endpoints using natural soils with varying physical and chemical parameters, to adequately assess the toxicity of chemicals.

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Chapter 7

Effect of soil properties and ageing on the toxicity of Cu for *Enchytraeus albidus*, *Enchytraeus luxuriosus* and *Folsomia candida*

7. Effect of soil properties and ageing on the toxicity of Cu for *Enchytraeus albidus, Enchytraeus luxuriosus* and *Folsomia candida*

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ABSTRACT

In the present study, the effect of the heavy metal copper chloride ($CuCl_2 2H_2O$) in soils freshly spiked (3 days) and aged (70±10 days) was studied in the test species *Enchytraeus albidus*, *Enchytraeus luxuriosus* and *Folsomia candida*. Up to nine soils were used: besides the OECD artificial and LUFA 2.2 natural standard soils, the others were selected based on the EURO-Soil approach, taking into account the effect of different soil parameters (pH, OM, grain size distribution, C/N). Additionally, the effect of the chloride ions was studied separately. The results revealed **1)** a **soil effect**, e.g. in *F. candida* EC_{50}s varied between 261.8 mg/kg in ESo5 soil and >1000 mg/kg in OECD soil; **2)** an **ageing effect**, mainly in *F. candida*, e.g. in the OECD soil toxicity was increased twice and in the ES7 approximately eight times with ageing while the enchytraeid species did not react differently after ageing; **3)** an effect of **chloride ions** on reproduction of the animals, however, this effect was independent of the exposure time; and **4) species** variation in terms of sensitivity, decreasing in the following order: *E. luxuriosus* > *E. albidus* >> *F. candida*. Differences in toxicity between enchytraeids and *F. candida* might be explained by the different exposure routes of uptake.

Keywords: Natural soils, laboratory tests, EURO-Soils.

1. Introduction

Terrestrial toxicity not only varies between species but the soil characteristics also greatly influence the effect concentration of metals by altering for instance the bioavailability (Lock and Janssen, 2001a). Due to reasons of practicability and comparability, it is mostly common to use in toxicity testing the OECD artificial soil (OECD, 1984) or the standard natural LUFA 2.2 soil. Nevertheless, current risk assessment procedures ignore that variation in soil properties results in substantial differences for uptake and effects in organisms in different soils (Peijnenburg, 1999a). Therefore, it is very important to study the effects of different soils characteristics in terms of a) the suitability of the test species and b) the influence on the toxicity of a certain toxic substance.

Here we tested the effects of the heavy metal copper on two groups of organisms, enchytraeids and collembolans. Organisms with different exposure routes like oligochaetes and arthropods should be used simultaneously to assess the environmental risk of metal contaminated soils (Lock and Janssen, 2003a). In addition, standardized test procedures are available for the selected test species, *Enchytraeus albidus*, *Enchytraeus luxuriosus* and *Folsomia candida*. The common long-term application of copper fungicides against pests results in soil contamination (Filser and Hölscher, 1997). Despite that Cu is an essential metal, if high dosages are present it becomes toxic to soil invertebrates. Among the reasons for selecting Cu as a test substance are also the fact that this is a well known substance and results from our studies can be compared with the Dutch (e.g. Peijnenburg *et al*., 1999a) and Belgian (Lock and Janssen, 2001b) data.

Ageing is an important issue to take into account: laboratory tests should mimic the most realistic situation and freshly spiked soils are not allowing the equilibration time that is required to resemble the common field situation. Incorporation of the effect of ageing in the environmental risk assessment of metal-contaminated soils may contribute to a more realistic assessment of the impact of metals on terrestrial ecosystems (Lanno et al. 2004). One of the problems is in terms of feasibility of the tests: the recommended aging period is of a minimum of 60 days (McLaughlin *et al.* 2002), a very long period when thinking about the urgent demand of results and the

need of experimental repetition. Therefore, the assurance of the need of such periods is very relevant and should be investigated.

2. Materials and Methods

Test Species

Two groups of organisms were used: Enchytraeids and Collembolans. The test species selected among the Enchytraeids were *Enchytraeus albidus* Henle, 1837 and *Enchytraeus luxuriosus* (Schmelz & Collado, 1999). *E. albidus* is one of the largest species of the genus *Enchytraeus* (adults reach 15 – 40 mm) while *E. luxuriosus* (adults reach 8 – 13 mm) is much smaller. Both species were maintained in laboratory cultures, being bred in moist soil (50% OECD soil, 50% natural garden soil), at 20ºC, kept in the dark and fed once a week with finely ground and autoclaved rolled oats (Cimarrom, Portugal). Details of the culturing process are given in Römbke and Moser (2002).

Among the Collembolans, *Folsomia candida* Willem 1902 (Collembola: Isotomidae) is the most commonly used test species. It is a blind, unpigmented euedaphic collembolan reproducing parthenogenetically (Hopkin 1997). This species is easily cultured in laboratory in a moistened substrate of plaster of Paris/charcoal (mixture 8:1) prepared according to Usher & Stoneman (1977). Organisms are maintained in laboratory at 20ºC in the dark and fed dried backer's yeast (*Saccharomyces cerevisae*). Synchronized cultures were established for the experiments by removing egg clusters from stock cultures into new culture vessels. Two days after the start of hatching, juveniles were transferred into a new vessel, where they got food and water. After approximately 10 days for *F. candida* the organisms were in the appropriate life stage to start the test.

Test Substance

The test substance, Copper chloride (di) hydrated – $CuCl_2 2(H_2O)$ – (MERCK, 99% pure), was added as aqueous solution to the soils in the following concentrations: 3.2, 10, 32, 100 and 320 mg/kg dry soil, for the enchytraeid testing, and 10, 32, 100, 320

and 1000 mg/kg dry soil, for the collembolan testing. These concentrations were selected on the basis of literature data (e.g. Løkke & van Gestel 1998; Lock & Jansen 2001b). The contamination of all test substrates was done by mixing the aqueous solutions of the chemical into the pre-moistened soil, each test concentration into the whole batch of soil for all replicates. After homogeneous mixing, sub-samples of the batch of soil were introduced into the test vessels.
Additionally, a "control chloride" was prepared to evaluate the effect of the chloride ions added simultaneously with the heavy metal $CuCl_2 2(H_2O)$. KCl (MERCK, 99.5% pure) was added in an aqueous solution resembling the amount of chloride added in the highest concentration with the copper compound.

Test procedures

The Enchytraeid Reproduction Test (ERT) was standardised in 2003 for the study of single chemicals as well as contaminated soils (ISO 2003; OECD 2003). Ten adult worms with well developed *clitellum* were introduced in a glass vessel, each containing 25 g of moist soil plus food supply (finely ground and autoclaved rolled oats; 0.5 mg for *E. albidus* and 0.25 mg for *E. luxuriosus,* being half of the amount supplied every week). Four replicates per treatment were used. The duration of the tests with *E. albidus* was six weeks: after three weeks the adults were gently removed and the soil was left for three additional weeks for juveniles to hatch and grow. The test duration for *E. luxuriosus* was 4 weeks and the adults were left in the vessels until the end of the test. At the end of the test, the organisms' were immobilisised with alcohol and coloured with Bengal red. After some hours the organisms are coloured and the soil solution was spread in a box and observed under the binocular for counting. Adult mortality and the number of juveniles were evaluated for both species.

Test procedures were as described in the ISO guideline 11267 for *F. candida* (ISO 1998). Ten organisms with an age of 10 - 12 days were placed in each test vessel, already containing the pre-moistened test soil and the food supply. Vessels were covered with a parafilm layer in which a few holes for airing were made. Food and water was replenished weekly. After four weeks the test ended and each test vessel

was filled with distilled water which was gently mixed with a spatula. Afterwards, juveniles and adults were floating on the surface. Due to the addition of a few drops of dark ink, a higher contrast between the white organisms and the black background was obtained. A digital photograph of the water surface plus the collembolans was made. The collembolans on the image could later easily be counted using the computer software SigmaScan Pro 5 (SPSS, 1999). As a result, a number is proposed by the software. Some replicates are randomly selected and the pictures are checked by hand, to validate the accuracy of the program. Adults and juveniles were easily distinguished by their size.

Test soils

The main properties of the test soils (pH, Organic Matter, Carbon/Nitrogen, Cation Exchange Capacity, maximum Water Holding Capacity, clay, silt and sand content) are given in Table 1. Their selection is described in detail by Römbke and Amorim (2004).

Table 1: Main characteristics of the tested soils and the relative EuroSoils (ES) properties: pH, OM, C/N, grain size distribution, CEC and maximum WHC (* means that the CEC was evaluated with uncertainty).

SOIL	pH ($CaCl_2$)	O.M. (%)	C/N	Clay (%)	Silt (%)	Sand (%)	CEC (mval/100g)	WHC (%)
Nat1	6.2	1.7	8.7	33	66	5	40.7	58.4
Hoh2	6.2	12.9	25.0	6	61	33	78.3*	73.9
Coi3	6.7	6.5	17.0	26	60	14	75.8	68.1
Sch3	5.4	4.1	10.4	23	45	32	68.5	67.4
LUFA 2.2	5.8	4.4	14.0	6	17	77	11.2	55
Mon4	6.5	2.5	9.7	11	77	12	20.7	53.2
Eso5	3.2	9.2	29.7	10	12	79	87.0*	100.1
ES7	4.4	11.5	14.2	19	35	46	5.0	80.6
OECD artificial	6.0	8.0	Ca. 40	10	10	80	45.8*	Ca. 90

OECD artificial soil (OECD, 1984) is constituted by 69% sand, 20% kaolin clay, 10% sphagnum peat and 0.3 to 1% of $CaCO_3$ for pH adjustment (6±0.5). LUFA 2.2 is a natural standard soil from Speyer (Germany). Relatively to the other codes given

for the natural soils, ES7 means EURO Soil 7, ESo5 means that the soil is a sample from the same site as the original ES5, the numbers mean that the soil is similar to a certain ES number and, the other codes represent the first three letters of the soil original place name: Nat1: Natzungen; Hoh2: Hohenlimburg; Coi3: Coimbra; Sch3: Schmallenberg; Mon4: Mönninghausen.

Not all soils were used in the three test species (Tab. 2) due to several reasons, e.g. not fulfilling the validity criteria (for details see Amorim *et al.*, 2004 a,b).

Experimental setup

The different soils were previously tested just as a control, to evaluate their suitability to a certain test species (for details see Amorim *et al.* 2004a,b).

A first set of experiments was conducted three days after the application of the test substance. A second set was performed 70 (±10) days after application, to allow chemicals to equilibrate. This experimental set up followed the recommendations of a workshop organised by the Society of Environmental Toxicology and Chemistry (SETAC) (McLaughlin et al., 2002), that recommended that three different evaluations are made: 1) 2 to 7 days after mixing the substance into the soil; 2) 60 days after the 2 to 7 days initial incubation of the substance into the soil; 3) same as 2) but additionally the soil is leached 2 to 7 days after mixing the substance into the soil. However, due to time and resource limitations the third option was not tested here. This 60 days period is referred as a transformation time and it is a trade-off between practical considerations and allowing a realistic amount of time for transformation reactions, with rapid reactions for metals typically having half-life values in the range of 1 - 100 days.

Statistical procedures

Three main hypotheses were tested, as follows:

1) The measured soil properties influence the toxicity of the toxic compound in two ways: by directly altering the exposure (e.g. due to different adsorption and bioavailability) or by adding another stress factor for the organisms in addition to the chemical.

2) Ageing is affecting the toxicity.
3) The chloride ions are affecting the results obtained with the toxic compound.

Different methods were used to test the hypothesis:

- Stepwise Multiple Regression models were performed, using the statistical software package SPSS 12.0 (SPSS, 2003), to quantify the relationship of the biological data with soil data. Data was normalised using log(X+1) transformation in the regression models, except for pH. Additionally, only the two extreme categories (sand and clay, excluding silt) of the three texture classes were used due to the interdependence of the individual parameters. Nevertheless, no statistically significant regressions were obtained.
- Analysis of variance (two way) were calculated using SigmaStat 2.03 (SPSS, 1997) to evaluate differences between each soil.
- Student T-test (SPSS, 1997) was used to analyse statistically significant differences between controls of each time of ageing and between control and control chloride.
- EC_{50s} and NOECs were calculated using the ToxRatPro program (ToxRat 2003).

3. Results

1) Freshly spiked *versus* Aged soils

In Figures 1-3 and Table 2-3 the results of the tests with the three species in the different soils are presented. Unfortunately, the number of invalid tests, in particular in the case of enchytraeids where the absolute number of tests was limited was high.

Regarding the results for *E. albidus*:

1) There was no significant effect of ageing on the adults in any of the soils tested (Two-way ANOVA).
2) In the OECD soil, there was a statistically significant effect of time (Two-way ANOVA: $F=62.850$; $df = 1$; $p< 0.001$) on the reproduction, in terms of

total number of organisms produced (maximum number of juveniles at day 3 around 300 and in aged soil around 140).

3) In the LUFA 2.2 soil, there was a statistically significant effect of the interaction of time and the toxic concentrations (Two-way ANOVA: $F_{1,6}$= 4.066; p=0.003) on the reproduction (the EC_{50} slightly decreased from 97 to 122 mg/kg). However, since the two controls were significantly different (T-Test: t=-3.111; df = 6; p = 0.021), the effect of time could not be confirmed.
4) No comparisons were made in the case of Coi3 soil due to the ambiguous results obtained after 3 days.
5) Comparing the soils, toxicity increased in the following order: OECD < Coi3 < LUFA 2.2. EC_{50}s in OECD and LUFA 2.2 soil differed by a factor of at least 3.

The results for *E. luxuriosus* were as follows:

6) There was no significant effect of ageing on the adults in any of the soils tested (Two-way ANOVA).;
7) In the OECD soil, there was a statistically significant effect of time (Two-way ANOVA: F= 8.625; df = 1; p = 0.005) and of the toxic concentrations used (Two-way ANOVA: F= 4.942; df = 6; p < 0.001) at the concentration of 320 mg/kg on reproduction (toxicity decreased by a factor of about 3). Since the controls between each time were significantly different (T-Test: t= 3.526; df = 6; p = 0.012), the effect of time could not be confirmed.
8) No comparisons can be made in the case of LUFA 2.2, Sch3 and Coi3 soil because of invalid ageing test results.

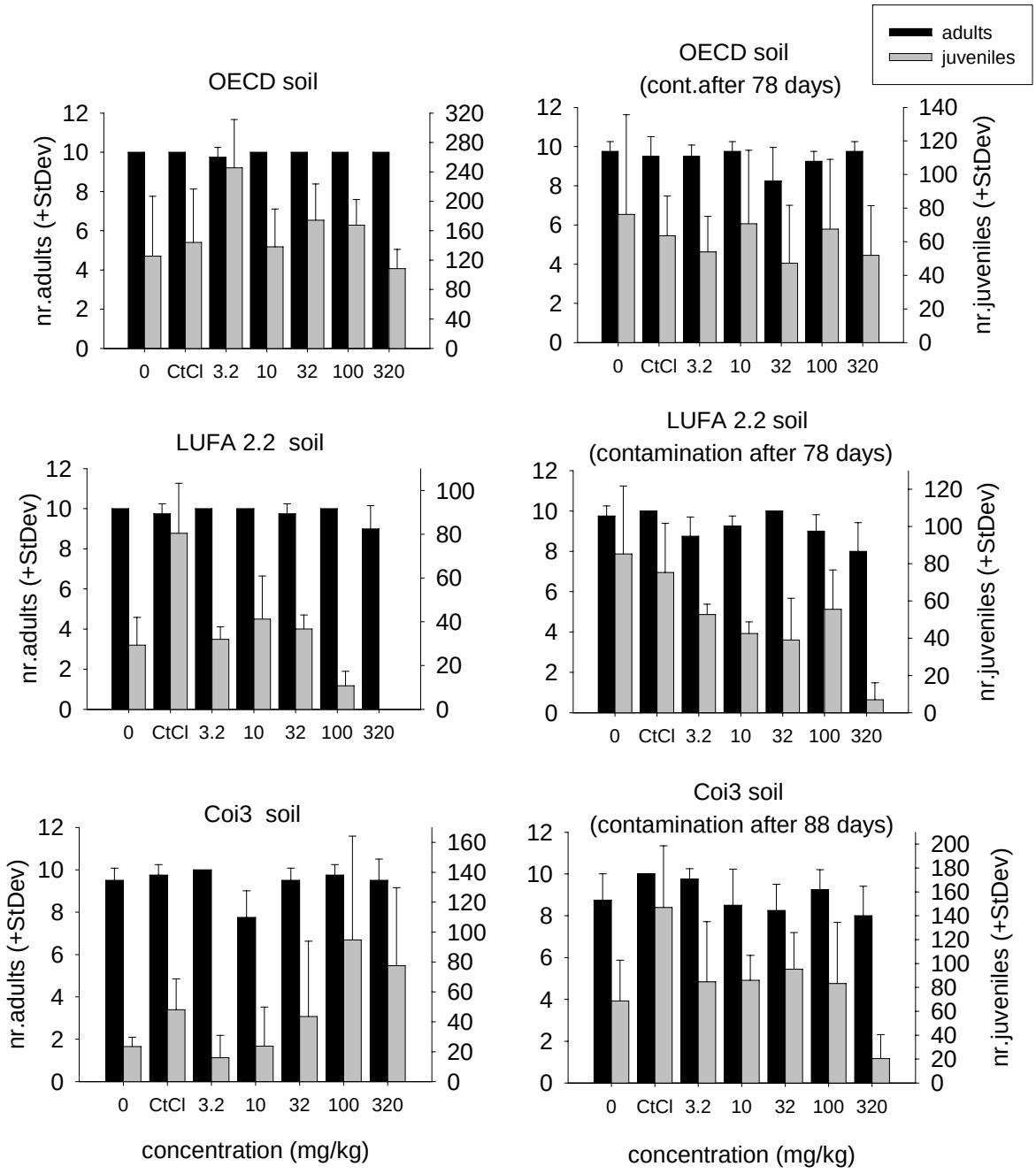


Figure 1: Exposure of *E. albidus* to $CuCl_2 2(H_2O)$ in freshly spiked and aged soils.

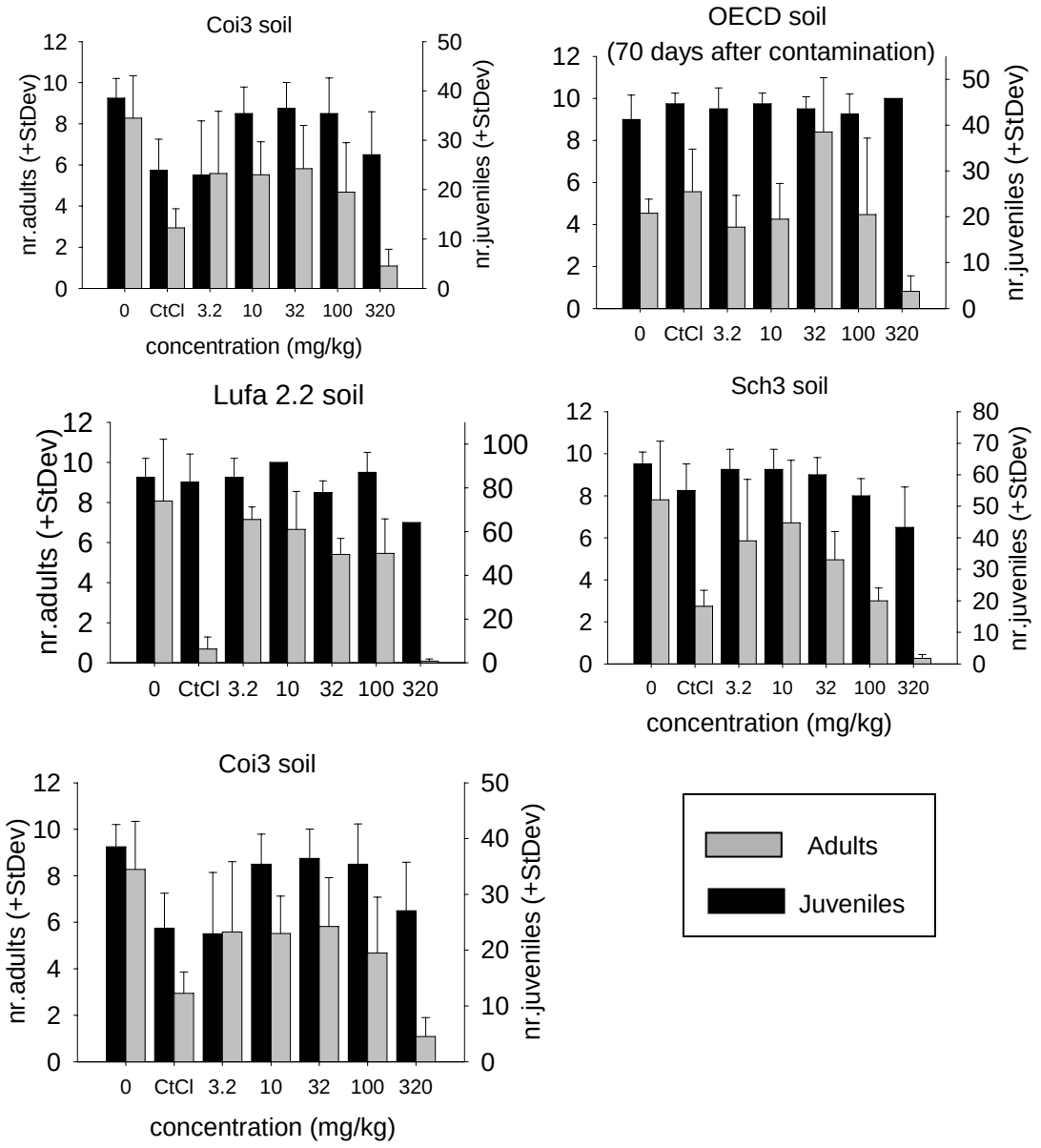


Figure 2: Exposure of *E. luxuriosus* to $CuCl_2 2(H_2O)$ in freshly spiked and aged soils.

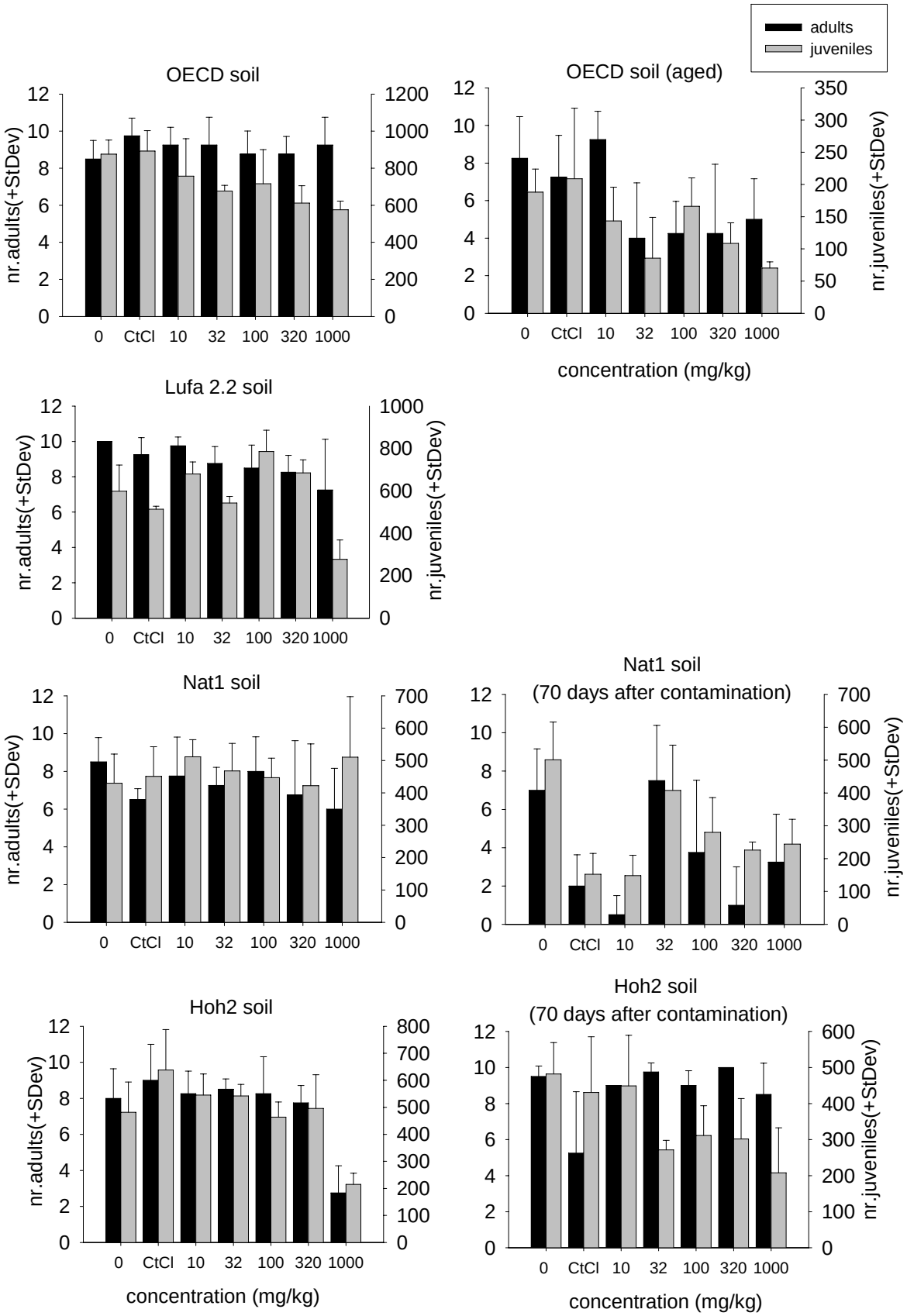
For *F. candida* it was found that:

9) In the OECD soil, there was a statistically significant effect of time (Two-way ANOVA: F= 505.417; df = 1; $p < 0.001$) and of the toxic concentrations used (Two-way ANOVA: F= 5.518; df = 6; $p < 0.001$) (1000/320 mg/kg ≠ CtCl), on the reproduction. Since the controls between each time were significantly different (T-Test: t= 16.23; df = 6; $p < 0.001$), the effect of time could not be confirmed.

10) In the Hoh2 soil, there was a statistically significant effect of time (Two-way

ANOVA: F= 24.697; df = 1; $p < 0.001$) and of the toxic concentrations used (Two-way ANOVA: F= 8.447; df = 6; $p < 0.001$) at the concentration of 1000 mg/kg on the reproduction.

11) In the Coi3 soil, there was a statistically significant effect of time (Two-way ANOVA: F = 231.140; df = 1; $p < 0.001$) and of the toxic concentrations used (Two-way ANOVA: F = 2.996; df = 6; $p < 0.001$) (32 mg/kg ≠ Ct) on the reproduction. Since the controls between each time were significantly different (T-Test: t= 3.857; df = 6; p = 0.008), the effect of time cannot be confirmed.
12) In the Mon4 soil, there was a statistically significant effect resulting of the interaction of time and the toxic concentrations (Two-way ANOVA: $F_{1,6}$= 5.253; $p < 0.001$) on the reproduction, but, since the controls between each time were significantly different (T-Test: t= 9.403; df = 6; $p < 0.001$) the effect of time could not be confirmed.
13) In the ESo5 soil, there was a statistically significant effect of time (Two-way ANOVA: F = 39.266; df = 1; $p < 0.001$) and of the toxic concentrations used (Two-way ANOVA: F = 57.610; df = 6; $p < 0.001$) (CtCl/320/1000 mg/kg ≠ 100/10/Ct/32 mg/kg) on the reproduction.
14) In the ES7, there was a statistically significant effect resulting of the interaction of time and the toxic concentrations (Two-way ANOVA: $F_{1,6}$= 4.944; $p < 0.001$) on the reproduction, but, since the controls between each time were significantly different (T-Test: t= 6.797; df = 6; $p < 0.001$), the effect of time could not be confirmed.
15) No comparisons can be made in the case of the LUFA 2.2 and the Nat1 soils.
16) The results concerning the adults are presented in Table 2. There was an effect of time in most of the tested soils, except in the Mon4, causing a decrease in the absolute number of juveniles produced.


adults
juveniles
OECD soil
OECD soil (aged)
Lufa 2.2 soil
Nat1 soil
Nat1 soil
(70 days after contamination)
Hoh2 soil
Hoh2 soil
(70 days after contamination)
nr.adults(+StDev)
nr.adults(+SDev)
nr.juveniles(+StDev)
0 CtCl 10 32 100 320 1000
concentration (mg/kg)

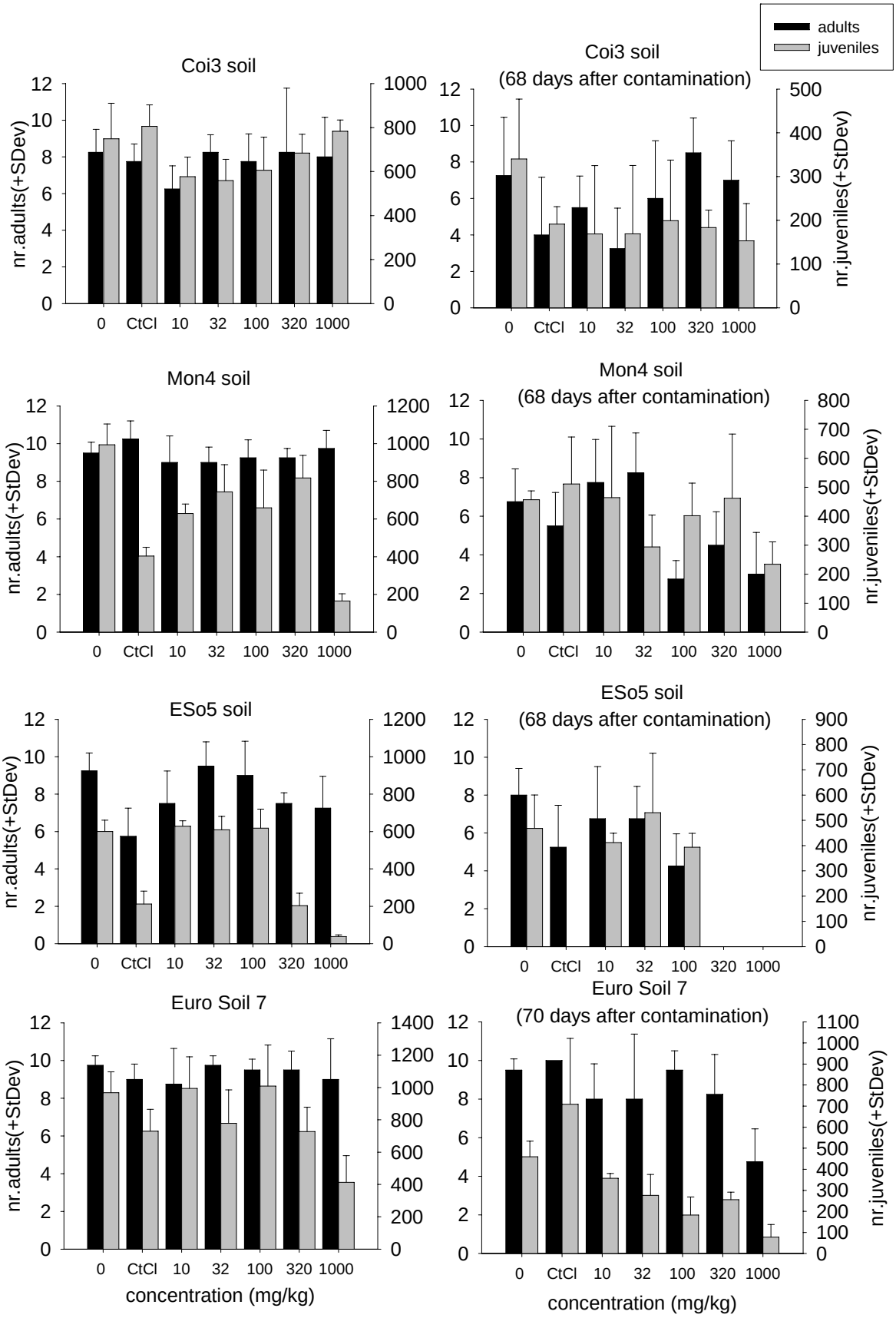


Figure 3: Exposure of *F. candida* to $CuCl_2\,2(H_2O)$ in freshly spiked and aged soils.

Table 2: Two Way ANOVA and T-test, relatively to the number of adults of *F. candida* in freshly spiked soil versus aged soil. (Conc.=Concentration).

Soil	Source of Variation	F	df	p	Effect
OECD	Time x Conc.	2.519	1,6	0.036	No effect between Ct: effect of time confirmed
Hoh2	Time x Conc.	6.936	1,6	<0.001	No effect between Ct: effect of time confirmed
Coi3	Time	9.566	1	0.004	No effect between Ct: effect of time confirmed
Mon4	Time x Conc.	5.441	1,6	<0.001	(T-Test: t= 3.051; df = 6; p = 0.002); no effect of time
ESo5	Time x Conc.	7.225	1,6	<0.001	No effect between Ct: effect of time confirmed
ES7	Time	6.199	1	0.017	No effect between Ct: effect of time confirmed
	Concentration	3.059	6	0.014	at the concentration of 1000 mg/kg

Despite that some tests were not valid the results with *F. candida* seem to indicate that toxicity increased after ageing. For example, EC_{50} values were lower by a factor of about two in the OECD and the ESo5 soils and by a factor of eight in the ES7 soil after ageing. Between soils, toxicity increased in the following order OECD/Nat1/Coi3 (value > 1000 mg/kg)) < LUFA 2.2 < Hoh2 < ES7 < Mon4 < ESo5. EC_{50}s varied between OECD/Nat1/Coi3 soil and ESo5 (freshly spiked), by a factor of about four, and between OECD soil and ESo5 (aged) by a factor of about six.

Several stepwise regression models were run but none gave results that allow a significant relationship between test species and soil parameters.

Table 3: EC_{50}s and NOECs from tests with *E. albidus, E. luxuriosus* and *F. candida* exposed to Copper chloride in freshly spiked (three days) soils and aged (70 ±10 days) soil. (Mort. = mortality; Rep. = Reproduction; n d = not determined due to mathematical reasons; x = test not valid).

Enchytraeus albidus

	EC_{50} (Cu)		**NOEC (Cu)**		**EC_{50} (aged)**		**NOEC (aged)**	
Soil	Mort.	Rep.	Mort.	Rep	Mort.	Rep	Mort.	Rep.
OECD	>320	>320	≥320	≥320	>320	>320	≥320	≥320
LUFA 2.2	>320	97	≥320	100	>320	121.8	≥320	100
Coi3	>320	n d	≥320	n d	>320	303.8	≥320	100

Enchytraeus luxuriosus

	EC_{50} (Cu)		**NOEC (Cu)**		**EC_{50} (aged)**		**NOEC (aged)**	
Soil	Mort.	Rep.	Mort.	Rep	Mort.	Rep	Mort.	Rep.
OECD	>320	64.8	≥320	3.2	>320	228.9	≥320	100
LUFA 2.2	>320	80.5	≥320	10	x	X	x	x
Sch3	>320	47.8	≥320	10	>320	X	≥320	x
Coi3	>320	90.8	≥320	32	>320	X	≥320	x

Folsomia candida

	EC50 (Cu)		**NOEC (Cu)**		**EC50 (aged)**		**NOEC (aged)**	
Soil	Mort.	Rep.	Mort.	Rep	Mort.	Rep	Mort.	Rep.
OECD	>1000	>1000	≥1000	10	n d	662.3	10	10
Lufa 2.2	>1000	986.6	≥1000	320	x	X	x	x
Nat1	>1000	>1000	≥1000	≥1000	x	X	x	x
Hoh2	868.8	947.7	320	320	>1000	n d	≥1000	10
Coi3	>1000	>1000	≥1000	≥1000	n d	n d	x	<10
Mon4	>1000	413.3	≥1000	320	n d	n d	32	320
ESo5	>1000	261.8	≥1000	100	74.5	161.5	32	≥100
ES7	>1000	793.8	≥1000	320	n d	101.5	320	<10

2) Control *versus* "control chloride"

No differences were observed between the tests with water (control) and those with chloride (control chloride) in the number of adults of both enchytraeid species and *F.*

candida. Statistically significant differences in reproduction were found for *E. albidus* in certain soils: LUFA 2.2 soil (freshly spiked) (T-test: t= -4.130; df=6; p = 0.006), and Coi3 aged soil (T-test: t=-2.830; df=6; p = 0.03) with the chloride causing a positive effect; in *E. luxuriosus*, similarly in certain soils (freshly spiked): LUFA 2.2 soil (T-test: t= 4.701; df=6; p = 0.003), Sch3 soil (T-test: t=3.502; df=6; p = 0.013) and Coi3 soil (T-test: t= 4.728; df=6; p = 0.003), the chloride was causing a decrease in the number of juveniles produced.

In *F. candida*, statistically significant differences in reproduction were found due to the chloride ions in certain soils: Nat1 aged soil (T-test: t =5.327, df=6; p = 0.002) Mon4 soil freshly spiked (T-test: t = 9.879; df=6; p<0.001) and ESo5 freshly spiked (T-test: t =8.485; df=6; p < 0.001) and aged (T-test: t = 7.058; df=6; p <0.001), causing a negative effect.

3) Comparison between species

The comparison between species is only possible in the cases of the OECD artificial and the LUFA 2.2 soil. Nevertheless, in these cases *F. candida* was less sensitive than the enchytraeids. Among the enchytraeid species, *E. luxuriosus* is more sensitive than *E. albidus*.

4. Discussion

1) Freshly spiked *versus* Aged soils and comparison between soils

Despite the low number of valid tests it seems that in the tests with the enchytraeid species the toxicity of copper in freshly spiked and aged soils was nearly the same. At a maximum, toxicity decreased over time by a factor of 3.5 in the test with *E. luxuriosus* in OECD soil. With *E. albidus*, no effect of ageing was observed. Similarly, Lock and Janssen (2002a) found no effect of ageing (8 weeks) on the toxicity of Zn (also an essential metal as Cu) for *E. albidus* in a test with OECD soil. The authors believe that the used clay type (kaolinite) may be responsible for this observation, since kaolinite has a low capacity of metal fixation in comparison with other clays such as bentonite and illite (Reddy and Perkins, 1974). However, the

same authors found that despite comparable total copper concentrations, pore-water copper concentrations were significantly higher in the freshly spiked soils compared to those in the historically contaminated soils (Lock and Janssen 2003b). In their study, pH decreased significantly after spiking, while in our tests only a slight decrease in pH (never larger than 0.5 and only in the highest toxic concentrations) occurred. Therefore, it is not clear whether the pore-water copper concentrations in the freshly spiked soils are higher because of the lower pH or because of the effect of ageing in the spiked soils.

On the other hand, in the case of *F. candida* ageing seems to have an effect on both adults and juveniles, increasing the toxic effect at the reproduction level. In the OECD soil, an increase by a factor of two and in the ES7 soil by a factor of approximately eight is occurring. Again, the lack of a complete data set hampers the generalisation of this observation but the fact that in all valid tests the toxicity increased makes it wise to perform tests with aged soils and *F. candida*. The same recommendation was made by Smit and Van Gestel, (1998) who studied the effect of Zn on *F. candida* after percolating metal-contaminated soils with water and the inclusion of an equilibration period prior to use. To achieve a more realistic exposure situation these procedures should be included into laboratory toxicity tests.

Between soils toxicity was increasing in the following order:

a) *E. albidus*: OECD > Coi3 > LUFA 2.2.

b) *E. luxuriosus*: Coi3 > LUFA 2.2 > OECD > Sch3.

c) *F.candida*: (OECD/Coi3/Nat1) < LUFA 2.2 < Hoh2 < ES7 < Mon4 < ESo5.

In the enchytraeid tests the differences in EC_{50} values were relatively small in most cases. This observation is at least partly caused by the fact that only in a limited number of soils valid tests could be performed due to the sensitivity of enchytraeid reproduction towards soil properties. In the only comparable data set found in the literature, an EC_{50} of 305 (235-374) mg/kg was obtained (±95% confidence intervals) in a test with *E. albidus* and OECD soil (Lock & Janssen 2002b). This result is in the same range as the results of our study.

However, major changes were obtained in the tests with *F. candida*. These differences are probably caused by an interaction of soil properties and Cu: for example, the low pH of the soil ESo5 is the factor causing the much higher toxicity in this soil in comparison to the other soils. This fact is in accordance with findings from Crommentuijn (1994), who tested pH levels from 7.3 to 3.1 and found a range of EC_{50}s for survival of 306 to 102µg Cd g^{-1} respectively, showing a tendency of increasing toxicity with lower pH. However, Sandifer (1996) did not observe an effect of pH on the reproduction of *F. candida* when exposed to metals (Cd, Cu, Pb, Zn). There was only an overall decrease in reproduction in the control samples at pH 5.0 and 4.5 in comparison to a pH of 6.0. Concerning other soil parameter than pH, no clear influence on toxicity can be established.

Furthermore, it is wise to reduce the amount of OM of the artificial soil to 2% since in the original OECD soil, containing 10% peat, the observed toxicity was lower than in most field soils (Wohlgemuth, 1990). Accordingly, Lock and Janssen (2001c) and Lock *et al.* (2000) found that instead of the content of clay and organic matter, the pH and the CEC were the most important parameters affecting Zn and Cd ecotoxicity: toxicity is decreasing with increasing pH and CEC. These authors state that CEC is a better parameter to estimate bioavailability and ecotoxicity than clay and OM quantity since CEC is a measure of the amount of available sorption sites and thus incorporates the clay, metal oxyhydroxides as well as OM of a soil. The question remains whether this statement is similarly true for Cu.

2) Control *versus* "control chloride"

No effect of chloride could be observed for adults in *E. albidus* and in *E. luxuriosus*, but the reproduction of the test species was affected in certain soils. Often the toxicity of metals in soil is determined by investigating the effects of metal salts without paying attention to the influence of the anionic partner of the investigated metal. Therefore, Schrader et al. (1998) evaluated the role of salt anions on the reproduction of *F. candida* by using a soil with a standard mixed salt solution, containing $CaSO_4$, $MgSO_4$, $MgCl_2$, KCl, and NaCl, applied at different concentrations. At higher salt concentrations, egg development was inhibited. Tests

with single salt solutions showed that this was due to the inclusion of NaCl (43.5 mmol/kg d.wt. soil) in the mixed salt solution. $CaCl_2$ tested separately also reduced egg survival. A comparison between a solution of salts and an elutriate of toxic waste containing heavy metals and similar salt ions showed a clear combination of salt effects and heavy metal effects. These studies indicate that chloride ions may interfere with the demonstration of toxic heavy metal effects. The authors concluded that when chloride salts are used to determine the toxicity of metal cations, additional tests with comparable anion solutions of non-toxic cations are needed, to clarify the results.

Finally, it remains an open question whether the increased toxicity after ageing as observed in several tests with *F. candida* has something to do with the effects caused by the chloride ions. Out of the two soils where this increase after 70 days was observed, only in ESo5 a very strong effect in the chloride control was visible. This means that probably a combination of low pH and chloride (similar to the combination of low pH and Cu) is responsible for this result. On the other hand, no explanation can be given for the strong effect in the chloride control of the soil Nat1 after ageing. However, this test is difficult to evaluate due to the fact that no dose-response relationship could be established.

3) Comparison between species

Species sensitivity to copper chloride decreased in the following order: *E. luxuriosus* > *E. albidus* >> *F. candida*. Differences in toxicity between enchytraeids and *F. candida* might be explained due to the different exposure routes of uptake: springtails are probably mainly exposed via the pore water, while dietary exposure is also important for worms (Lock and Janssen, 2003a). In this way, it was confirmed what could be expected: Enchytraeids were more affected than collembolans. Several studies show the higher sensitivity of oligochaetes towards copper in comparison to arthropods, e.g. Didden & Römbke (2001) for enchytraeids and Spurgeon *et al.* (1994) for earthworms.

4) General Discussion

Only few studies focusing on the effect of **different soil properties** on the **toxicity of Cu** for enchytraeids and collembolans are available.

In 1992, Van Gestel had concluded that many factors affect sorption to soil and uptake in organisms, and therefore extrapolation between soils seemed not yet possible. Later, Van Gestel *et al.* (1995), in a review on the influence on soil characteristics on the toxicity of metals for soil invertebrates, concluded that pH is the most important factor followed by soil organic matter content and cation exchange capacity. However, for each metal another combination of these factors seems to determine bioavailability. It was therefore not possible to derive general rules for the extrapolation of toxicity data between soils (Van Gestel, 1997). Moreover, the bioavailability of metals in soil seemed to fluctuate with time (Forge *et al.*, 1993). Vijver *et al.* (2001) studied the impact of soil properties in 16 Dutch field soils on the accumulation of heavy metals in *F. candida*. The authors confirmed that the bioavailability of metals depends on the metal, the soil properties and the species in question. Different patterns in accumulation of metals were found for essential metals (Cu, Zn) versus cadmium and lead. The authors suggest a composite uptake. Internal body concentrations of Zn are largely unaffected by external Zn concentrations, and the same trend is found for Cu. The organisms are able to maintain their internal concentration at a fixed level, independent of the external concentrations. In general terms, Cd and Pb uptake by *F. candida* is strongly associated to total metal pools and metal binding phases of the soils, such as carbonate clay and oxyhydroxydes. These findings show that biological diversity in uptake patterns exists between soil inhabitants. Solid soil phases are more important in the uptake process of springtails than expected based on formulae derived for soft-bodied oligochaete species and plants, which were influenced more strongly by pore-water characteristics. The latter results were obtained by Peijnenburg *et al.* (1999a,b, 2000) who found that the effects on soft bodied species and plants were strongly associated with metal pools in the pore water and in the $CaCl_2$-extractable fraction. A high proportion of the variation of tissue residues was explained by pH. Concluding, literature data on uptake and toxicity experiments carried out in OECD soil cannot be used in a straightforward manner to predict effects of metals in field soils.

Lock and Janssen (2001a) assessed the influence of soil type on cadmium ($CdCl_2$ $2H_2O$) availability in a standard artificial soil, a sandy and a loamy field soil. The authors could not evaluate the influence of soil parameters on the bioavailability on the basis of the conducted experiments nor by including literature data. *E. albidus* was the most sensitive species, followed by *E. fetida* and *F. candida.* Furthermore, they revealed that the acute ecotoxicity of Cu was mainly determined by pH and OM content (significant effect) and CEC (highly correlated). They could also show that the LC_{50}s for *E. albidus* exposed to metals varied over more than two orders of magnitude, depending on the composition of the artificial soil (Lock *et al.*, 2000; Lock and Janssen, 2001b). Later they studied the influence of copper ($CuCl_2$ $2H_2O$) and other metals on *E. albidus* in OECD soil (Lock and Janssen 2002b), and observed that the concentration-response relationships were steeper for the essential elements (Zn and Cu) than those obtained with the non-essential elements (Cd and Pb). Finally, Peijnenburg *et al.* (1999a) tested *E. crypticus* in 20 Dutch field soils contaminated with Cd, Cu, Pb and Zn. Multivariate expressions that describe uptake rate constants and bioaccumulation factors as a function of soil characteristics were derived. pH and CEC were the most important parameters, but these were differing with each metal. Unfortunately, relatively to Cu, concentrations on organisms after exposure did not differ significantly from body concentrations in the culture and, in view of this apparent regulation, the Cu data was considered irrelevant in this study. Hence, no relation to soil properties could be established for Cu. Thus, it seems to be doubtful whether reliable correction factors can be derived for the influence of soil properties on the toxicity of copper to soil invertebrates as it has been proposed for Cd in agricultural soils (Wohlgemut *et al.* 1990).

5. Conclusions

Our results confirm that soil properties affect the toxicity of Cu on enchytraeids and collembolans (**soil effect**). EC_{50}s were varying in each species from soil to soil, reaching factors of around 3 in *E. albidus* and about 6 in *F. candida*, although there was no relation with any specific soil parameter among the tested soils. Accordingly,

the precautionary values for copper as laid down in the German Soil Protection Ordnance (BBodSchV; 1999), different according to the three soils texture classes: 20, 40 and 60 mg/kg (total concentration), were set as a threshold for a "no-effect-level" in sandy, silt and clay soils. *F. candida* results showed an important effect of **ageing**, increasing the toxicity in a maximum by a factor of eight in the ES7 soil. The effect of the chloride ions, added simultaneously with the copper, was causing effects at the reproduction of *E. luxuriosus* and *F. candida* in certain soils and interestingly was changing with ageing in certain cases. Additionally, **species sensitivity** differed, Enchytraeids being more sensitive than *F. candida*. The literature research, relative to the influence of soil properties on the toxicity of metals shows that there are still no conclusive answers relative to these issues. Apparently, toxicity is changing with the soil, the metal and the species.

6. Acknowledgements

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Chapter 8

General Discussion and Conclusions

8. General Discussion and Conclusions

During this study several problems were raised that can be summarized as follows:

(1) Tackling the heterogeneity of soils in ecotoxicological testing is a very complicated problem and, still, a very urgent and demanding one. Obviously the number of natural soils potentially suitable for ecotoxicological testing is so high that the results of such tests would be impossible to compare and thus the data would be useless for regulatory purposes. So, the first step in this process must be a reduction in the number of potential test soils. Fortunately, it is possible to find natural soils belonging to the individual classes of the original Euro-Soils, the so called SIM(ilar)-Soils. This seems to be so far the most equilibrate criterion. Some practical problems are worth mentioning:

A) The classes have to be re-defined when more practical information with a higher number of natural soils has been investigated. Most problematic is the fact that the individual values can differ when different batches of the same soil from the same site are characterised. Speaking more generally, any classification based on fixed trigger values is problematic since in reality a continuum of soils exists. In the long run, the approach presented here has to be replaced by a more flexible system based on multi-variate statistical methods.

B) Up to now, the EURO-Soils as well as the SIM-Soils have been defined purely by soil properties. However, since the SIM-Soils are defined to be used as substrates for biological tests the reaction of the main test species has to be taken into consideration when fixing the details of the SIM-Soils classes.

Some soils cannot be used with (at least some) of the standard test species, e.g. *Enchytraeus albidus* in very acid tundra soils. In such cases new tests have to be developed – or at least existing tests have to be modified in a way that, for example, an acidophil oligochaete species can be tested. When accepting the SIM-Soil approach in general, several detailed questions have to be clarified. In the case of testing individual chemicals it is unlikely that the OECD artificial soil will be discharged completely in the future. Instead, artificial soil could be used for testing

on a first tier (e.g. comparable to the "base set" in aquatic testing), probably with a reduced amount of organic matter. In addition, it might serve as an external control to secure the quality of the individual test system. SIM-Soils are used in the standard invertebrate tests but the required number, which changes according to the tiers of the whole test strategy, has to be fixed. Depending on the results of the tests of the first tier or on the area where the test chemical will be applied, additional soils can be required. Due to the "open" definition of SIM-Soils their use is very flexible. To keep the whole SIM-Soil approach practical and also to get acceptable results, it is highly recommended that sufficient guidance for their use is provided before implementing the SIM-Soils.

The (2) Identification of the ecological requirements of the most important terrestrial ecotoxicological test species, based on literature studies, gives some very important information, but by far not enough for the number of proposed soil types (a minimum of about half a dozen is necessary to cover the most important soil classes in Europe). It became clear that standard test species, so far being mainly tested in OECD artificial soil or the natural standard soil LUFA St. 2.2, can also be tested in many natural soils. Soil arthropods (collembolans, mites) are, as expectable, less sensitive towards soil properties then soft-bodied oligochaete worms. Still, in many cases, research is needed to define more clearly the ecological requirements of the standard test species. Additionally, research is needed to provide alternative test species necessary to cover certain soils (e.g. sandy acid soils). While such soils have often been classified as "extremes" and thus are neglected, one should not forget that such soils are very common in, for instance, Scandinavia or Northern Canada. In fact, the same statement is true for most non-temperate and/or non-agricultural soils world-wide.

To evaluate the effects of different soil types on organisms, and its interaction with chemical substances, several effect levels were studied. The (3) Avoidance behaviour of *Enchytraeus albidus* was found to be appropriate for the study of screening effects. *Enchytraeus albidus* (and, referring to literature data, also *E. crypticus*) seems to be a suitable test organism for avoidance testing. LUFA 2.2 soil appears to

be the best choice as a control for substrate or chemical testing instead of OECD artificial soil, and so it may be used as a control in further similar bioassays. Avoidance tests are useful as screening tools for the assessment of potentially contaminated soils or of chemicals in soils. In addition, they are valuable to evaluate the influence of soil properties on these worms. Provided that a larger data base concerning the relation between avoidance and mortality/reproduction data is available, an avoidance test could be a time-saving alternative to long term tests. Due to these reasons and notwithstanding the need for further testing using other chemicals and soils, this test should be standardized and recommended for effect evaluation along the lines already discussed for the Earthworm Avoidance Test.

The (4) Effects of different soil types (alone and in combination with the herbicide Phenmedipham) on the enchytraeids *Enchytraeus albidus* and *Enchytraeus luxuriosus* was studied at the reproduction level. From the developed experiments one can conclude that soil properties limit the use of test species: enchytraeids showed a high sensitivity to changes in soil parameters and were mainly affected by low pH. Therefore, soil type is an important issue when discussing which species can be used for risk assessment and which effects can be expected due to extrapolation to the biotic community. More data using more soils and species are required to understand the effect of soil properties in soil toxicology. Nevertheless, it was clear that certain soil properties such as OM and WHC or pH, CEC, C/N and clay content did interact with the chemical and the organisms. In the present study, EC_{50}s in enchytraeids changed by a factor of 9 for juveniles and nearly 30 for the adults of *E. luxuriosus* (maximum values; slightly lower values were found for *E. albidus*), which shows how important the test soil can become for the environmental risk assessment of chemicals. In this context also the further usage of OECD artificial soil has to be discussed.

To have different groups of organisms being tested, the (5) Effects of different soil types (alone and in combination with the herbicide Phenmedipham) on the collembolans *Folsomia candida* and *Hypogastrura assimilis* was also studied at the reproduction level. The most important result of this study is that important soil

properties in a wide range do not limit the use of *F. candida* in ecotoxicological standard tests. However, soil had an important influence on *F. candida* when tested with Phenmedipham; i.e. the EC_{50}s of juveniles changed by a factor of approximately 10. Clearly, juveniles prefer soils with a high C/N ratio, while their preferences relatively to other soil properties are less clear. More data using more soils and species are required to understand the effect of soil properties in soil ecotoxicology. *H. assimilis* showed to be a more sensitive species to different soils than *F. candida*, although, the inherent high variability of results in *H. assimilis* and the less feasible and accurate extraction and counting procedures were acting as negative factors. Therefore, this species cannot be recommended for ecotoxicological standard tests unless technical improvements are made. However, the variability caused by the sexual reproduction behaviour will remain. The further role of OECD artificial soil in ecotoxicology should be discussed. Test results gained with this soil were often those showing the lowest toxicity. Despite the fact that this observation is based on tests with just one chemical, it is clear that the relatively high organic matter content is the main cause of an often reduced bioavailability of a test substance and thus its toxicity. For example, a reduction of the peat content (e.g. to 5% peat) would probably be more realistic while still being acceptable for soil invertebrates. In addition, the use of natural peat in a standard substrate should be re-considered, since different kinds of peat are known to induce changes in the fate of chemicals and the behaviour of organisms. Still the possibility to get well comparable results by using this artificial substrate is worthwhile, but it is simply not representative of the diversity of natural soils (and might underestimate toxicity). However, it might serve as an external control (probably with a reduced amount of organic matter) to secure the quality of the individual test system. The results presented here show that soil toxicity testing should not rely solely on tests with artificial soil, but should include assays with reproductive endpoints using natural soils with varying physical and chemical parameters to adequately assess the toxicity of chemicals.

Since toxicity not only varies between species and soils but also with the chemical substance, the (6) Effect of soil properties alone and in combination with copper (directly after spiking and after ageing) on *Enchytraeus albidus*, *Enchytraeus*

luxuriosus and *Folsomia candida* were studied. The assessment of the environmental risk of metals in soils is hampered by at least three problems: firstly, it is often ignored that the different soil properties can have a substantial effect on the organisms directly as well as indirectly on the interaction between a metal and an organism (i.e. the toxicity). Secondly, ageing of the metal in soil, also depending on the different soil properties, is rarely taken into account. Furthermore, metals are commonly added to soils as a combination with salts, ignoring the effect of such anions. The results revealed a soil effect, e.g. in *F. candida* EC_{50}s varied between 261.8 mg/kg in ESo5 soil and >1000 mg/kg in OECD soil; an ageing effect, mainly in *F. candida* (e.g. in the OECD soil toxicity was increased twice and in ES7 approximately eight times with ageing) while enchytraeid species did not react differently after ageing; an effect of chloride ions on reproduction of the animals (however, this effect was independent of the exposure time, e.g. after a 70 days ageing period toxicity could either increase or diminish); and species differentiation in terms of sensitivity, decreasing in the following order: *E. luxuriosus* > *E. albidus* >> *F. candida*. Differences in toxicity between enchytraeids and *F. candida* might be explained by the different exposure routes of uptake: springtails are probably mainly exposed via the pore water, while dietary exposure is probably also important for worms: Enchytraeids were more affected than Collembolans. This difference is in accordance with literature data showing a higher sensitivity of oligochaetes than arthropods towards copper exposure.

Summarizing, the issue of soils diversity and consequences for organisms and testing procedures is a problem yet to be solved in its completeness. Nevertheless, steps were made in the direction of finding a solution and/or alert to this problem.